Development of eco-friendly methods for monitoring and regulating the box tree pyralid, *Cydalima perspectalis* (Lepidoptera: Crambidae), an invasive pest in ornamentals

Dissertation

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„Diese Menschen sitzen sämtlich wie die Raupe auf einem Blatte,
jeder glaubt, seines sei das Beste und um den Baum
bekümmern sie sich nicht.“

HEINRICH VON KLEIST

Content

Summary

As a monophagous herbivorous species that is native to Asia, the Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) is one of the accidentally introduced non-native species that successfully survived, reproduced and became established in Europe. The aesthetic damage and total loss of *Buxus* is not only limited to local plantings, but also extends widespread in urban areas as well as to the natural *Buxus* stands, which are already affected or remain highly endangered. The objective of this investigation was the implementation of an effective, eco- and user friendly method for regulating *C. perspectalis* and preserving unaffected *Buxus* plants, based on usable biocontrol agents as well as on an effective monitoring system.

The occurrence of wild populations of *C. perspectalis* and seasonal moth flight activity was recorded by pheromone and light traps between 2012 and 2015 in Hessia (GER). Both trap systems were found to be suitable for monitoring *C. perspectalis* that is required to allow the proper timing of control measures. Two main flight periods were identified, which indicate the existence of two generations. Basic data on the sex ratio (on average 43% ± 10% females) and the appearance of the melanic phenotype (on average 14% ± 2.9%) were collected.

The ability of eight parasitoid *Trichogramma* species to parasitize and locate *C. perspectalis* eggs was assessed in the laboratory. Despite all tested species examined and oviposited into exposed eggs, resulting parasitation rates were low and ranged between 4% and 20% for most of the species. Only *T. dendrolimi* parasitized significantly more eggs with rates above 40%. Potentially egg defensive mechanisms were suggested that may impede higher parasitization. Thus, no specialized *Trichogramma* species had been found yet for a practicable inundative release.

The beneficials *Chrysoperla carnea* Stephens (Neuroptera: Chryspidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae) preyed on *C. perspectalis* eggs in experimental studies on prey acceptance and prey location on *Buxus* plants. *C. carnea* preyed on average 89% offered eggs during the prey acceptance bioassay and 49% offered eggs during the prey location on the *Buxus* plant within 72 h. In contrast, *O. majusculus* preyed only 41% and 13%, respectively. Therefore, the conservation of natural enemies as *O. majusculus* and the inundative release of beneficials such as *C. carnea* can be considered as supporting tools in the biological control of *C. perspectalis*.

Susceptibility of *C. perspectalis* to the prospective biological control agents Neem (NeemAzal®-T/S) and Entomopathogenic nematodes (Nemastar®) was assessed in laboratory bioassays and field trials. When NeemAzal®-T/S treated leaf discs were consumed by larvae, a significant effect on vitality and feeding activity was noted after 14 days of exposure (47%-62%

mortality; 10% of larvae were still feeding). Larvae (2nd and 4th developmental stage) demonstrated high susceptibility to Nemastar® (50 EPN per larva caused 100% mortality after 7 days). Both agents were much less reliable in their efficacy in field trials in comparison to the constant effectiveness of *Bacillus thuringiensis*. Their use is only possible in smaller areas, if individual feeding damage will be observed and proper surveillance will be considered.

Potential repellent and toxic properties of 13 plant oils and extracts on egg deposition of *C. perspectalis* female moths and larval mortality were investigated in the greenhouse and the laboratory. When *Thymus vulgaris* L. and *Malaleuca alternifolia* M. & B. treated leaf discs were offered to larvae, a significant effect on feeding deterrence, survival and larval weight was noted. *T. vulgaris* was the most effective oil considering repellent and toxic effects. Its effective lethal concentration (LC_{50}) was determined to be 1% (v/v) at 72 h.

None of the tested control options alone provide an opportunity for the effective, eco- and user friendly regulation of *C. perspectalis*. The application of products based on *B. thuringiensis* is still the only highly effective and available biological control method but new approaches dealing with moth behaviour manipulation are promising, since the concept leads to the preservation of unaffected *Buxus* plants. In conclusion, the regulation of the invasive Box tree pyralid *C. perspectalis* still remains a major challenge for all concerned parties, even 10 years after its introduction.

Zusammenfassung

Als eine monophage herbivore Art die aus Asien stammt, ist der Buchsbaumzünsler *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) eine der versehentlich eingeführten nicht-einheimischen Arten, die erfolgreich überleben, sich fortpflanzen und auch etablieren konnte. Der ästhetische Schaden und das Absterben von *Buxus* beschränkt sich nicht nur auf lokale Anpflanzungen, sondern erstrecken sich auch flächendeckend in städtischen Gebieten sowie auf die natürlichen Buchsbestände, die bereits betroffen oder stark gefährdet sind. Ziel dieser Forschungsarbeit war die Implementierung einer wirksamen umwelt- und benutzerfreundlichen Pflanzenschutzmethode zur Regulierung von *C. perspectalis*, einschließlich der Erhaltung von noch nicht befallenen Buchsbeständen, auf der Basis von anwendbaren biologischen Methoden sowie eines effektiven Monitoring Systems.

Das Auftreten von *C. perspectalis* Populationen sowie der jährliche Verlauf der Flugaktivität wurden in den Jahren 2012-2015 mit Pheromon- und Lichtfallen in Hessen (DE) erfasst. Beide Fallensysteme erwiesen sich als dazu geeignet ein effektives Monitoring von *C. perspectalis* durchzuführen, welches notwendig ist, um Kontrollmaßnahmen gut terminieren zu können. Es wurden zwei Haupt-Flugphasen identifiziert, die auf die Existenz von zwei Generationen schließen lassen. Grundlegende Daten zum Geschlechtsverhältnis (durchschnittlich 43% ± 10% Weibchen) und zum Vorkommen des melanisierten Phänotyps (durchschnittlich 14% ± 2,9%) wurden erhoben.

In Laborstudien wurde die Fähigkeit von acht *Trichogramma* Arten untersucht, *C. perspectalis* Eier zu parasitieren und auf der Pflanze zu lokalisieren. Obwohl alle Arten die Eier inspiziert haben und Eiablagen erfolgten, war die daraus resultierende Parasitierung bei fast allen Arten gering und lag zwischen 4% und 20%. Nur *T. dendrolimi* parasitierte signifikant mehr Eier und erreichte Parasitierungsraten von über 40%. Es ist denkbar, dass Abwehrmechanismen vorliegen, die möglicherweise eine höhere Parasitierung verhindern. Bisher konnte keine spezialisierte *Trichogramma* Art gefunden werden, die für einen praktikablen inundativen Einsatz in Frage kommt.

Die Nützlinge *Chrysoperla carnea* Stephens (Neuroptera: Chryspidae) und *Orius majusculus* Reuter (Heteroptera: Anthocoridae) erbeuteten *C. perspectalis* Eier in Laborstudien zu Akzeptanz und der Lokalisierung auf der Buchspflanze. *C. carnea* erbeutete im Durchschnitt 89% der angebotenen Eier in den Akzeptanzversuchen und 49% der angebotenen Eier wurden innerhalb von 72 h auf der Buchspflanze gefunden und erbeutet. Im Gegensatz dazu erbeutete *O. majusculus* nur jeweils 41% und 13%. Daher kann die Erhaltung natürlicher Gegenspieler wie *O. majusculus* und der inundative Einsatz von Nützlingen wie *C. carnea* als unterstützendes Instrument in der biologischen Regulierung von *C. perspectalis* in Betracht gezogen werden.

Die Anfälligkeit von *C. perspectalis* Larven gegenüber den aussichtsvollen biologischen Mitteln Neem (NeemAzal®-T/S) und Entomopathogenen Nematoden (Nemastar®) wurde in Labor- und Freilandversuchen ermittelt. Nach 14 Tagen der Exposition und des Fraßes von Blattscheiben die mit NeemAzal®-T/S behandelt wurden, konnten signifikante Effekte auf die Vitalität (47%-62% Mortalität) und die Fraßaktivität (10% der Larven fraßen noch) festgestellt werden. Larven des 2. und 4. Entwicklungsstadiums zeigten eine hohe Empfindlichkeit gegenüber Nemastar®(100% Mortalität nach 7 Tagen bei 50 Nematoden pro Larve). Beide Mittel waren in ihrer Wirksamkeit in Freilandversuchen, im Vergleich zur konstanten Wirksamkeit von *Bacillus thuringiensis,* viel weniger zuverlässig. Ihre Verwendung ist nur in kleineren Bereichen möglich, wenn individuelle Fraßschäden beobachtet werden und eine ordnungsgemäße Überwachung berücksichtigt wird.

Mögliche repellente und toxische Effekte von 13 Pflanzenölen und -Extrakten auf die Eiablage von *C. perspectalis* Weibchen und die Mortalität von Larven wurde in Gewächshaus- und Laborversuchen untersucht. Nach der Exposition und des Fraßes von Blattscheiben die mit *Thymus vulgaris* L. und *Malaleuca alternifolia* M. & B. behandelt wurden, konnten signifikante Fraß hemmende Effekte sowie Effekte auf die Vitalität und das Larvengewicht festgestellt werden. *T. vulgaris* war das wirksamste ätherische Öl unter Berücksichtigung von repellenten und toxischen Eigenschaften. Die effektive lethale Konzentration (LC_{50}) belief sich nach 72 h auf 1% (v/v).

Keine der untersuchten Pflanzenschutzmaßnahmen allein bietet die Möglichkeit einer effektiven, umwelt- und benutzerfreundlichen Regulierung von *C. perspectalis*. Die Anwendung von Produkten, die auf *B. thuringiensis* basieren, ist nach wie vor die einzige hochwirksame und verfügbare biologische Kontrollmethode, aber neue Ansätze, die sich mit der Verhaltensmanipulation der Weibchen beschäftigen, sind vielversprechend, da das Konzept auf dem Schutz noch nicht befallener Buchspflanzen basiert. Als Schlussfolgerung kann man sagen, dass die Regulierung des invasiven Buchsbaumzünslers *C. perspectalis* nach wie vor eine große Herausforderung für alle beteiligten Parteien ist, sogar 10 Jahre nach seiner Einführung.

General introduction

Invasive species

The history of establishing non-native species in Europe was initiated thousands of years ago when humans had begun to settle down and reclamation of land as well as domestication took place. Non-native species have been introduced in indigenous environments for the cultivation of new plants and animals to secure the basis for people's life and nutrition. Vavilov (1951) described in the 1930s twelve world centers of the origin of our recent cultivated plants. Thus, the native home of our grain crops is the Central Asiatic Center and the world's potential sources of orchard fruits are concentrated in the Middle East, the native home of the apple, grape, pear, cherry, pomegranate, walnut, quince, almond and fig. The discovery of the New World in the 16th century brought vegetables like tomatoes and potatoes from the South American Center to our fields and the availability of new foods increased rapidly. Therefore, non-native species build an important basis for our crop diversity today. In fact, the majority of species used in agriculture, forestry and fisheries are originally non-native species (Wittenberg & Cock 2001). However, many of the once imported species are no longer regarded as foreign because they have been integrated into our native natural and cultural landscape. In Europe, terms were defined to describe originally foreign species that do not naturally occur in a given area. The generic term neobiota is used to summarize organisms which were introduced after 1492 to such a new given area and became established under the direct or indirect assistance of humans. Non-native plant species are referred to as neophytes, accordingly, animal species as neozoans and fungal species as neomycetes. Plant species that occurred in Europe before 1492 are defined as archeaophytes (Nentwig et al. 2007).

However, remote trading relationships developed and during the 20th century human trade, tourism, transport and travel over the world have dramatically increased. The 20-year average of growth in the volume of world merchandise trade amounted to 5.3% per year (WTO 2016). This increasing movement of goods and people greatly increased the transcontinental transport of organisms. There are several entry pathways by which natural geographic barriers can be intentional or accidental overcome until today (Wittenberg & Cock 2001). If the climatic conditions are similar to those in their native range and no natural enemies occur, or if they are particularly competitive, introduced species are able to settle down, reproduce and spread rapidly in new environments (Wittenberg & Cock 2001). Furthermore, invasions will be facilitated by climate change. It may also be a significant, long-term driver enhancing invasive species introduction and spread. Strong effects are expected and already recognized through rising atmospheric carbon dioxide concentrations,

warmer temperatures, greater nitrogen deposition, altered disturbance regimes and increased habitat fragmentation (DAISIE 2009; Dukes 2011).

According to Williamson and Fitter (1996a, 1996b) non-native species that have already been introduced are capable of reaching different levels of invasion success (introduced, established and pest). They specified the "tens rule", a rule of thumb that declare that a respective amount of only 10% of species are able to pass the different steps in the invasion process. Thus, only a small proportion of introduced species have the potential to survive, reproduce and become established without human intervention, further, only 10% of these established species will become invasive and a serious pest. The term invasive alien species is defined as a species that is non-native to the ecosystem under consideration and whose introduction causes or is likely to cause economic or environmental harm or harm to human, animal or plant health (Richardson & Pyšek 2004; NISC 2006). The term pest additionally describes harmful species that show an increase in presence, abundance, density, geographic extent and causing negative impacts to humans, their resources or the environment but pest species can also be native to the concerned countries (Richardson & Pyšek 2004).

Non-native species are the major threat to biodiversity (EEA 2007). In general, four major groups of invasive species impacts can be classified. These are impacts on (1) biodiversity, (2) ecosystem services (3) human health and (4) economic activities. This includes for example genetic effects on indigenous species or their extinction, disruption of plant communities and cultural landscapes, reduction of harvest mainly in agriculture, forestry and fisheries, effects on the infrastructure, transportation ways and water availability as well as the possible action as vectors for new diseases (GISP 2007; Kenis et al. 2009; EEA 2012; DAISIE 2017). Occurring threats are usually irreversible and their handling causes enormous costs (Pimentel 2011). One example of a highly destructive and cost intensive invasive neozoan is the Zebra mussel *Dreissena polymorpha* Pallas (Veneroida: Dreissenidae) which is one of the 100 worst invasive species in Europe (Lowe et al. 2000; DAISIE 2017; EPPO 2017c), and became a serious pest in the United States, after introduction by ships travelled from Europe. It was estimated that this species caused $ 100 million costs per year by damage and associated control in 2000 (Pimentel 2011). Additionally, it is responsible for many significant ecological impacts throughout freshwater and estuarine water bodies in both Europe and the United States (Karatayev et al. 1997; Millane et al. 2008).

The introduction of more than 12000 species has been documented for Europe. About 15% of these alien species cause economic damages and harm to biological diversity (DAISIE 2009; EU 2014). For Germany more than 2000 non-native species were counted (861 terrestrial plants, 732 terrestrial

invertebrates, 198 aquatic species, 149 terrestrial fungi and 121 terrestrial vertebrates) whereby the individual harm effects could also be different. Terrestrial plants and invertebrates are the two taxonomic groups with most species causing negative impacts (DAISIE 2017).

Thus, all over the world, there are numerous international and national organizations that increasingly network while dealing with the wide and complex field of invasive alien species, with the aim of creating a basis for collective actions. Actions are the implementation of frameworks, programs, agreements and regulations, to define research priorities and policy goals as well as to provide management options and the gathering and exchange of information, for example by research-based databases. Elaborating management plans are helpful to summarize objectives on prevention, the early detection, eradication and the control of invasive species and develop strategies that enable a proper handling. The main European organizations, conventions and data bases are shortly described in Box 1. Unfortunately, joint implementation and accordingly the successful prevention of spreading invasive species leave much to be desired. Europe does not have a particularly good record in managing alien species, with only 34 species (primarily vertebrates) successfully eradicated from one or more regions. This is probably due to the limited awareness of the public and decision makers, the inadequacy of the legal framework and the scarcity of resources (Genovesi 2005).

Box 1 Main European organizations, conventions and data bases working on invasive species.

- **The International Union for Conservation of Nature (IUCN)** is the world's largest membership Union that provides public, private and non-governmental organizations with the knowledge and tools that enable human progress, economic development and nature conservation. The IUCN established the **Species Survival Commission (SSC).** This is a science-based network of more than 10000 volunteer experts working together in more than 140 Specialist Groups, Red List Authorities and Task Forces. One of these groups is the **Invasive Species Specialist Group (ISSG)** as a global network of scientific and policy experts on invasive species. **The Global Invasive Species Database (GISD)** is managed by the ISSG and was developed as part of the global initiative on invasive species led by the formerly **Global Invasive Species Programme (GISP).**
- **Convention on Biological Diversity (CBD)** is an international convention signed by 168 members to develop national strategies (Strategic Plan for Biodiversity 2011–2020) for the conservation and sustainable use of biological diversity to prevent the immigration of new invasive species, as well as to better control and, if necessary, eliminate already migrating species. It is often seen as the key document regarding sustainable development.
- **Regulation (EU) No 1143/2014** of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. This Regulation sets out rules to prevent, minimize and mitigate the adverse impact on biodiversity of the introduction and spread within the Union, both intentional and unintentional, of invasive alien species. Based on the Commission Implementing Regulation (EU) 2016/ 1141 of 13 July 2016 a list of invasive alien species of Union concern was adopted pursuant to Regulation (EU) No 1143/2014. This regulation also requires differentiated arrangements for the prevention and management of the introduction and spread of invasive alien species, hence the **German Federal Agency for Nature Conservation** (Bundesamt für Naturschutz, BfN) has published two **management manuals on the handling of non-native invasive species** in Germany (Scheibner et al. 2015; Schmiedel et al. 2015).
- The **International Plant Protection Convention (IPPC)** is an international plant health agreement of 185 partners, approved by the **Food and Agriculture Organization of the United Nations (FAO)** Conference that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. **International Standards for Phytosanitary Measures (ISPM)** as "Guidelines for Regulating Wood Packaging Material in International Trade" were adopted by the IPPC to reduce the risk of importation and spread of quarantine pests. The **European and Mediterranean Plant Protection Organization (EPPO)** is an intergovernmental organization under the IPPC, responsible for cooperation and harmonization in plant protection. More than 280 Standards for the efficacy evaluation of plant protection products (PP1) and for pest risk analyses (PRA) of invasive alien plants have been approved by EPPO and included in a database.
- The **Centre for Agriculture and Biosciences International (CABI)** is an international not-for-profit organization that provides information and applies scientific expertise **by the Invasive Species Compendium (ISC)** to solve problems in agriculture and the environment.
- **Delivering Alien Invasive Species Inventories for Europe (DAISIE)** is an EU-funded research project that developed the first pan-European inventory of Invasive Alien Species delivered via an international team of leading experts in the field of biological invasions, latest technological developments in database design and display, and an extensive network of European collaborators and stakeholders.

Introduction and distribution of *Cydalima perspectalis* in Germany and Europe

The monophagous Box tree pyralid *Cydalima perspectalis* (Lepidoptera: Crambidae) (Figure 1) is native to the humid subtropical regions of East Asia (EPPO 2016). Its occurrence in Asia is known from China (Walker 1859), Korea (Gu 1970) and Japan (Inoue et al. 1982). In addition, Hampson (1896) mentioned its occurrence in the Indian state Himachal Pradesh but this was the only record for India and therefore it is listed as unreliable and currently absent to India (CABI 2017). *C. perspectalis* adults and larvae were firstly detected in Europe (Southern Germany) in 2007. In the vicinity of the port of trans-shipment in Weil am Rhein (Baden-Württemberg) first notifications of feeding damage on *Buxus* plants were mentioned (Billen 2007). The already massive occurrence and the strong infestation in 2007 led to the conclusion that the new species must have already been introduced in 2005 or 2006; most likely by an accidentally import with a containership from China as a hitchhiker (Billen 2007; Krüger 2008). Until today, the general opinion is that the main introduction pathway is the international trade of *Buxus* plants and the fast spread of *C. perspectalis* is most likely caused by the repeated supply of infested plants (Leuthardt et al. 2010; van der Straten & Muus 2010; John & Schumacher 2013). Gninenko et al. (2014) published that reaching the Caucasus region can also be attributed to the fact that plants have been imported from Italy for landscaping the Olympic village in Sochi. From first report to present, the species spread across the European Union continuously raised and can be observed in more than 20 countries (Table 1).

Figure 1 *C. perspectalis* larva (left) and adult moth (right) on *Buxus*.

Table 1 Distribution (first records) of *C. perspectalis* in Europe.

Year	Country	First detection	Reference
2006	Germany	South Hessia, Baden-Württemberg	Billen (2007), Krüger (2008)
2007	Switzerland	five locations near Basel	Käppeli (2008), Leuthardt et al. (2010)
2008	France	Alsace	Feldtrauer et al. (2009)
	Netherlands	four different locations	Muus et al. (2009)
	United Kingdom	Southern England	Korycinska and Eyre (2009), Mitchell (2009), Salisbury et al. (2012)
2009	Austria	Mattes/Vorarlberg	Perny (2010)
	Liechtenstein		Aistleitner (2010), Slamka (2010)
2010	Romania		Iamandei (2010), Székely (2011)
2011	Belgium		Casteels et al. (2011)
	Italy		Bella (2013), Griffo et al. (2012)
	Hungary	West Hungary	Sáfián and Horváth (2011)
	Turkey	European part, Istanbul	Hizal et al. (2012), Öztemiz and Doğanlar (2015)
	Czech Republic		Šumpich (2011)
	Slovenia		Seljak (2012)
2012	Croatia	North, Arboretum Opeka, Vinica	Koren and Črne (2012), Matošević (2013)
	Slovakia	near Bratislava	Pastorális et al. (2013)
2013	Greece	Northern Greece, Thessaloniki	Strachinis et al. (2015)
	Russia	Black Sea coast (Sochi)	Gninenko et al. (2014), Karpun and Ignatova (2013)
	Spain	province of Pontevedra (Galicia, Iberian Peninsula)	Pérez-Otero et al. (2014)
2014	Bulgaria	Eastern part	EPPO (2016)
	Bosnia and Herzegovina		Ostojić et al. (2015)
	Montenegro and Serbia		Ostojić et al. (2015)
2015	Ukraine	Crimea	EPPO (2016)
	Luxemburg		Hellers and Christian (2016)

In Europe, *C. perspectalis* experiences only small competition by other herbivores or mortality by natural enemies (Wan et al. 2014) and the European climatic conditions facilitated to reproduce and establish. Moreover, based upon a climate model, Nacambo et al. (2014) suggested that *C. perspectalis* would probably continue its spread across all of Europe, except for Northern Fenno-Scandinavia, Northern Scotland and high mountain regions. Its spread is limited by an insufficient number of degree-days above the temperature threshold in the North, whereas it is limited by the absence of a cold period in the south that is necessary to resume diapause. Thus, damage (Figure 2 and Figure 3) is likely to be higher in Southern and Central Europe where the moth is able to complete at least two generations per year (Nacambo et al. 2014).

Figure 2 Characteristic scarping damage by neonate larvae (left) and leaf damage by older larvae (right).

Figure 3 *Buxus* hedge (length 5 m x width 1 m x height 2.5 m) in autumn 2015 (left) and in spring 2017 (right). Total loss after infection by *C. perspectalis*. No regulation measures were taken.

Biological invasions proceed through a series of transition probabilities (i.e. introduction, establishment, spread and impact) and each of these present opportunities for management. Commonly, the first step in dealing with non-native species is to distinguish the harmful species

from the harmless via Pest Risk Analyses (PRA). Collecting important data on the species biology and ecology, introduction pathways and distribution sites is necessary to evaluate the invasive potential of an alien species and avoid the possible introduction of a harmful organism into specific areas (Wittenberg & Cock 2001; Lodge et al. 2016). During this process, species are placed in certain categories or lists that allow or restrict the importation of the assessed organism. In 2007, *C. perspectalis* was added to the EPPO Alert List. Species recorded in the EPPO Alert List have been selected by the EPPO Secretariat or proposed by EPPO member countries, might presenting a risk to the EPPO region. Such species are assessed through the prioritization process to determine whether they should fall into the List of Invasive Aliens or the Observation List to provide early warning. Since no particular international action was requested by the EPPO member countries and no Pest Risk Assessment was conducted, *C. perspectalis* was deleted from the Alert list in 2011 (EPPO 2017b). It was not suggested to classify *C. perspectalis* as a quarantine pest and no common approaches were sought for its eradication. Thus, *C. perspectalis* rapidly established and became invasive. The dimensions of potential and recent economic and ecological damage were not foreseen; on the contrary, since it is causing damage to an ornamental plant, it was even trivialized. But threats to *Buxus* can cause high economic costs and the natural *Buxus* stands including its ecosystems are highly endangered and already partially destroyed (Kenis et al. 2013), therefore threats are not only a problem in ornamentals as is seemed to be for early affected countries.

In addition, another invasive organism causes serious damage to *Buxus* plantings. It is the very harmful fungus *Cylindrocladium buxicola* Henricot (Hypocreales: Nectriaceae) (Henricot & Culham 2002) an ascomycete fungus causing bark cancers, leaf necrosis and twig dieback, up to the decease of whole plants. It was mentioned in Europe for the first time in 1994 by a tree nursery in Hampshire, UK. Since 1998, "box blight" is widely rife in the UK and New Zealand. In 2000 it was found in Belgium and a rapid spread occurred to France, Netherlands, Ireland and Italy. Until now more than 20 European Countries as well as USA (2011) and Canada (2012) are concerned (EPPO 2017a). First proofs for Germany were detected for Northern Germany (Lower Saxony and Northrhine-Westphalia) in 2004. In the same year it was added to the EPPO Alert List but was deleted in 2008 because no particular international action was requested by the EPPO member countries (EPPO 2017b). Today, it is the most harmful threat to *Buxus* plants. It is well known, that larval feeding (Figure 2) by *C. perspectalis* facilitates the fungus infection. Thus, a combination of both pests can often be observed, and if it is the case, total loss of the plant (Figure 3) highly likely occurred. Since the introduction, the distribution in Germany has been very advanced for both organisms (Box 2).

Box 2 Distribution of *C. perspectalis* and *C. buxicola* in Germany (Göttig & Herz 2016b).

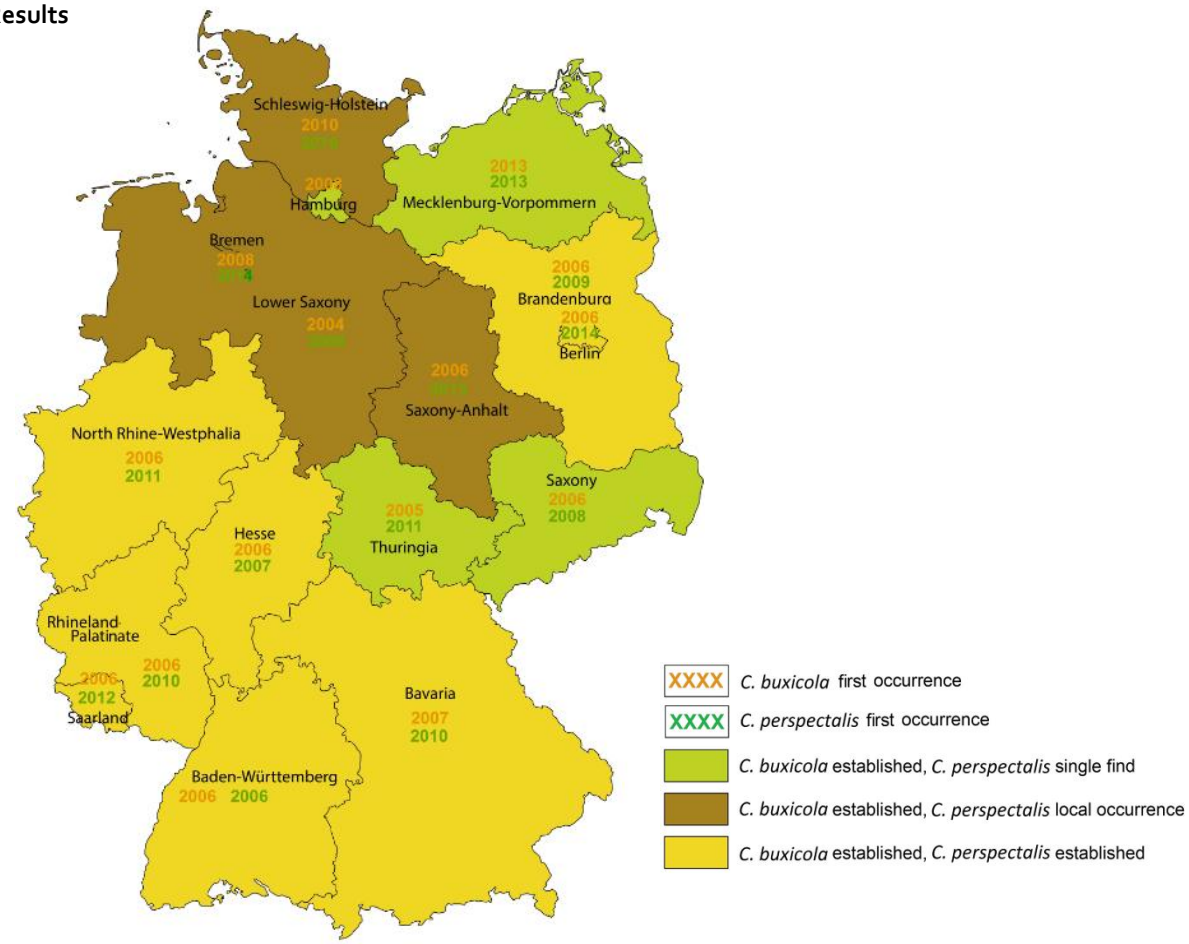
Introduction

The recent distribution of *C. perspectalis* and *C. buxicola* in Germany was ascertained via a developed questionnaire in 2016. The intention was to assess how far *C. perspectalis* and *C. buxicola* have been spread in Germany and whether there are regions left without infestation.

Material and methods

Addressees were the 16 plant protection services of the German Federal States, the Palace Departments of Germany as well as selected nurseries, which are offering *Buxus* plants. Ten questions were made about the time and degree of establishment, the damage intensity and the ecological and economic harm as well as applied and possible measures. 37 questionnaires were answered and taken into account.

Results



	Cydalima perspectalis	***Cylindrocladium buxicola***
Spread	has already appeared in all the federal states at least as a single find	has already appeared in all the federal states
Degree of establishment	established in the south-west and in Brandenburg	already established in all federal states
Damage intensity	infestation is assessed as low to moderate	infestation is assessed as moderate to strong
Applied and possible measures	mainly mechanical and chemical, but also biological measures are mentioned	mainly mechanical and preventive chemical measures are mentioned
Ecological and economic harm	The potential economic damage is assessed more strongly than the ecological and the future of *Buxus* as a popular purchased decorative plant is questioned	

Biological characteristics of *Cydalima perspectalis*

Originally, the species was described as *Phakellura perspectalis* by Francis Walker in 1859. In the course of time, it has been placed in various spilomeline genera including *Diaphania* Hübner, *Palpita* Hübner, *Glyphodes* Guenée and the monotypic *Neoglyphodes* Streltzov. After the introduction into Europe it was identified as the Box tree moth *Diaphania perspectalis* Walker (Lepidoptera: Pyralidae) by Dr. Klaus Sattler, a specialist for microlepidoptera of the Natural History Museum in London but after analyzing its morphology and phylogeny it was transferred to the genus *Cydalima*, a more reasonable and verifiable generic placement for the Box tree moth (Mally & Nuss 2010).

C. perspectalis is an herbivorous insect that is highly monophagous and specialised on the plant genus *Buxus* sp. (van der Straten & Muus, 2010; Leuthardt & Baur, 2013; Wan et al., 2014). The life cycle of these crepuscular and nocturnal moths occurs completely on *Buxus* plants, were oviposition, larval development, pupation and overwintering take place (Figure 4).

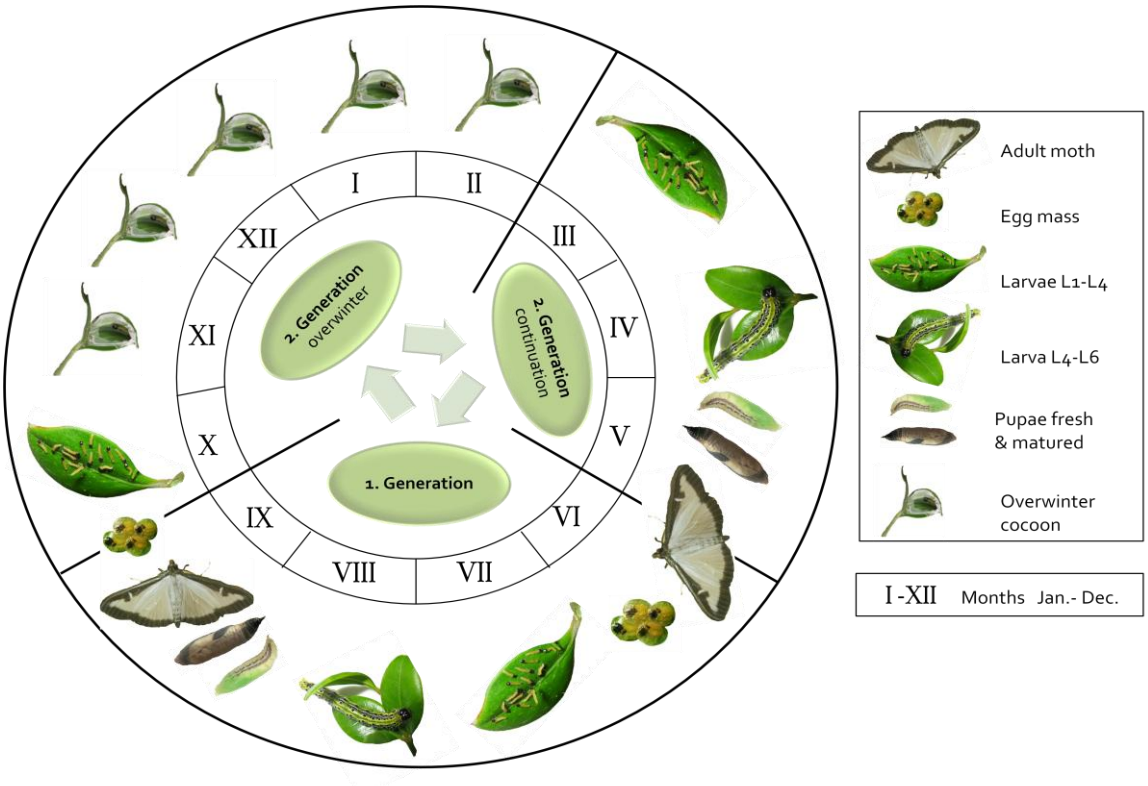


Figure 4 Seasonal life cycle of German *C. perspectalis* populations based on monitoring data (Chapter I) and occurring developmental stages adapted from personal field observations.

Adult females lay their eggs in masses, consisting of three to approximately 50 eggs (personal observation) mostly on the lower leaf surface after mating and dispersal. Larvae are going to hatch out approximately within a few days and undergo a feeding period of several weeks, depending on the occurring abiotic factors. At the beginning small larvae feed aggregated, causing damage on the exterior leaf layer and form loose nests in the plant. Later on, they spread and feed solitary on the foliage, typically sparing the vein and often additionally attacking the bark. Six to seven larval instars are usually passed until the pupation. Larvae spin a cocoon among a few leaves and evolve to a pupa from which the moth will emerge and the cycle begins anew. Larvae of the next generation appear in autumn. They feed until the third larval instar and overwinter in a closely spun cocoon between a few leaves. Diapause is induced by day length of less than 13.5 h (Xiao et al. 2011) and includes an obligate duration of at least 1.5 months at cold temperatures (Nacambo et al. 2014). In spring, feeding activity restarts. In Germany there are two to three occurring generations (Albert & Lehneis 2010; CABI 2013; Zimmermann 2014; Göttig & Herz 2017). The biological characteristics of Asian *C. perspectalis* populations do not differ from own observations of German populations in the laboratory (Table 2). The theoretical developmental zero examined for Japanese populations is 10.1°C and 365 day degree for the larval period (Maruyama & Shinkaji 1987).

Table 2 Duration [days] of developmental stages at different temperatures compared between Asian[1] and German[2] populations.

Temp.	**Eggs Asian**	**First to seventh larval instar Asian**							**Pupae Asian**	**Complete Asian**
		L1	**L2**	**L3**	**L4**	**L5**	**L_6**	**L7**		
15°C	15 ± 0.6									
20°C	7 ± 0.2									
25°C	4 ± 0.2	3	3	3	3	3	8		10 ± 0.36	
30°C	3 ± 0.1									
Temp.	**Eggs German**	**First to seventh larval instar German**							**Pupae German**	**Complete German**
		L1	**L2**	**L3**	**L4**	**L5**	**L_6**	**L7**		
15°C	14 ± 0.8	8	11	12	19	36	-	-	-	could not complete
20°C	7 ± 0.0	4	6	5	6	7	13	14	17 ± 1.0	55 ± 9.6
25°C	5 ± 0.8	3	3	3	4	5	7	6	9 ± 0.5	36 ± 10.8

[1] (Wan et al. 2014) [2] (Göttig 2012)

It is known that different types of wing colour varieties exist in *C. perspectalis* populations. The typical morph is white with a dark brown margin and small characteristically crescent-shaped white marks on it. The body is white with a brownish abdominal segment. Body and wings of the melanic morph are almost uniform dark brown with the exception of the two white marks also described by Sáfián and Horváth (2011) and Székely (2011). In addition, an intermediate phenotype exists with an extra brown margin at the forewings, also recognized by Pan et al. (2011) in China. The melanic morph can already be identified at the pupal stage.

Cultural, ecological and economic importance of Boxwood (*Buxus* sp.)

Buxus as evergreen perennial phanerophyte (subgroup nano-phanerophyte) is a rare semi-shade plant occurring in oak forests or also in the beech grove of south-exposed slopes and growing on moderately dry, humid but also stony soils mostly in forests (Oberdorfer et al. 2001).

The origin of *Buxus* is located in the late Cretaceous period, based on analyzes of fossil pollen findings. It was concluded that *Buxus* already occurred in the northern Gondwanaland 80 million years ago and thus, its history is older than the history of mankind (van Trier & Hermans 2007). Since the Tertiary, *Buxus* has been represented in Western and Northern Europe (Di Domenico et al. 2012). A connection with the history of mankind dates back to the year 4000 BC. It was depicted on grave walls of Egyptian dignitariesin and there are also references to *Buxus* in the Old Testament. It was used for cultivation in the oldest gardens of China and served as key component of Roman planting schemes. *Buxus* plants are one of the most popular ornamental shrubs with great cultural significance. Many old folk customs have grown up around *Buxus*, such as the attribution of protective properties against the devil and being part of numerous apotropaic rituals. Even today, *Buxus* serves as a landmark for numerous cultural centers. The plant was also be used as a timber for tools, weapons, flutes and violins (van Trier & Hermans 2007). Until today, investigations are conducted on the potential of *Buxus* alkaloids as medicine, for example against human tumor cells (Bai et al. 2016).

Besides the cultural meanings, the rare natural *Buxus* populations have huge ecological importance. Potential ecological effects may occur due to serious threats on the rare natural *Buxus* stands. These are *B. sempervirens* L. in Central and Southern Europe, mainly occurring as understory of broadleaf forests or woodlands, garrigues and calcareous grasslands in the Pyrenees and Southern France as well as the more isolated stands around the Jura Mountains, in Central France and the southern European Peninsulas. In addition, minor stands can be located in Britain, Belgium, Luxembourg,

Germany, Sardinia, Montenegro and Kosovo (Di Domenico et al. 2012). Furthermore, *B. balearica* Lam. in the Mediterranean area and *B. colchica* Pojark, in the Caucasus, especially in the forests of Georgia are highly endangered (Matsiakh 2016). Most of these stands are natural habitat types, which demand classification as special areas of conservation and are now seriously endangered due to the arrival of *C. perspectalis.* It can be assumed that no *Buxus* species or variant exists, which is not serving as potential host plant for *C. perspectalis,* even if there were different injury levels and minor variances detected in nutritional input of different *Buxus* species to *C. perspectalis* larvae (Maruyama 1992, 1993). Destroying natural *Buxus* populations will have severe consequences on the plant species itself as well as on the functioning of unique forest ecosystems as a whole, firstly by the change of the ground covering vegetation due to increased exposure to sunlight as already have been observed after the destruction of the *Buxus* forest in the Nature Reserve of Grenzach-Whylen in Germany in 2010 (John & Schumacher 2013).

The species is grown as evergreen ornamental plant that takes clipping and holds its shape like no comparable plant in its native area and elsewhere in temperate regions. Three *Buxus* species and hybrids of these species are commonly used in the nursery and landscape trades: Littleleaf Boxwood *B. microphylla* Siebold & Zucc., Common Boxwood (*B. sempervirens* L.) and Korean Boxwood (*B. sinica* var. *insularis*) (Niemiera 2012). Since the invasion of *C. perspectalis* in Europe, outbreaks occurred on ornamental box trees in nurseries, cemeteries, historical monuments, public parks and private gardens. Economic effects were even trivialized since it is mainly causing damage to an ornamental plant. But thousands of plants in private gardens, cemeteries, public parks and palace grounds are affected and huge costs are caused by feeding damage and associated control as well as in the case of replacement plantings.

Objectives

In general, the aim of this thesis was to set up an effective, eco- and user friendly strategy for regulating *C. perspectalis* based on the implementation of usable biocontrol agents as well as on a monitoring system. The concept is illustrated in a schematic representation of the thesis (Figure 5), which comprises its objectives and an assignment of the outcome.

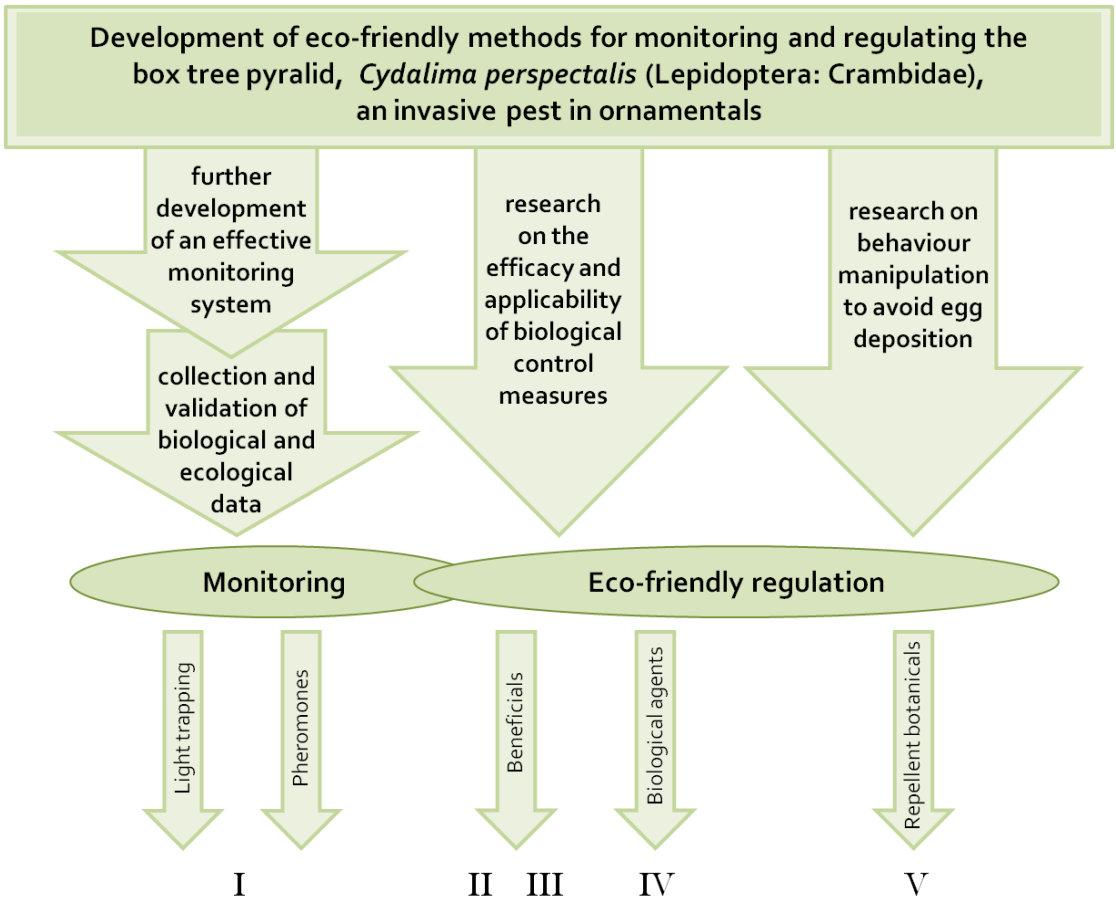


Figure 5 Schematic representation of thesis objectives and assignment of the outcome.

The present thesis consists of five scientific chapters, addressing different objectives in regard to the biology and biological control of *C. perspectalis*. The particular objectives of the manuscripts were in detail:

(I) Observations on the seasonal flight activity of the Box tree pyralid *Cydalima perspectalis* (Lepidoptera: Crambidae) in the Rhine-Main Region of Hessia.

Monitoring is an important component when dealing with invasive species, according to assess the spread, population levels and the seasonal flight activity per year. The emphasis of this study was to observe *C. perspectalis* populations for consecutive years with two monitoring systems, pheromone and light trapping to find out exact information on the seasonal flight activity patterns and whether there are differences in the temporal flight sequence of the sexes and of the two major morphological varieties. Comparison of different pheromone baits in field was also included.

(II) Are egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) promising biological control agents for regulating the invasive Box tree pyralid, *Cydalima perspectalis* (Lepidoptera: Crambidae)?

Egg parasitoids prevent hatching of larvae, thus reducing the risk for defoliation by larval feeding. This study was addressed to find a specialized *Trichogramma* species that accepts *C. perspectalis* eggs as a suitable host and is able to locate eggs on *Buxus*. Because *Trichogramma* wasps can already be mass-reared and produced for inundative releases, they might be an environmentally and user friendly tool with the potential to reduce *C. perspectalis* populations effectively.

(III) Prey acceptance and location of *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) eggs by *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae)

Egg predators have probably not to deal with toxins like dibasic alkaloids accumulated in the larval body. This investigation should clarify to which extent the commercially available beneficials *Chrysoperla carnea* and *Orius majusculus* do prey on *C. perspectalis* eggs. As polyphagous predators they were taken additionally into consideration to be potential predators of *C. perspectalis* and a suitable tool for supporting its biological control.

(IV) Susceptibility of the Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) to potential biological control agents Neem (NeemAzal®-T/S) and Entomopathogenic nematodes (Nemastar®) assessed in laboratory bioassays and field trials.

Eco-friendly preparations are needed which should be accessible, affordable, easy usable and suitable for the application in public areas, e.g. cemeteries, monasteries and public parks. The aim of this study was to assess the potential of common biological control agents, which are already commercially available on the market and bystander friendly to a great extent.

(V) Repellent and toxic properties of plant oils and extracts on *Cydalima perspectalis* Walker (Lepidoptera: Crambidae).

As a strictly monophagous herbivore, *C. perspectalis* reacts especially on physicochemical properties of *Buxus* plants. The aim was to find a repellent substance with toxic properties, which could serve to reduce oviposition and the overall risk for defoliation of *Buxus* plants by larval feeding. Experiments

were conducted in order to manipulate these properties and provide answers to the following questions: (i) does the application of plant substances deter females from oviposition; (ii) do essential oils with potential repellent properties additionally affect *C. perspectalis* larvae and if it is the case (iii) does a correlation between essential oil concentration and larval response (mortality) occur.

Chapter I. GÖTTIG S. & HERZ A. (2017) *Journal of Cultivated Plants*, 69 (5) 157-165

Observations on the seasonal flight activity of the box tree pyralid *Cydalima perspectalis* (Lepidoptera: Crambidae) in the Rhine-Main Region of Hessia

Abstract

The seasonal activity of wild populations of the invasive box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) was observed between 2012 and 2015 in the Rhine-Main region in Hessia. The moth flight was detected by a light trap (2012–2014) and several pheromone traps (2013–2015) at two locations. Two main flight periods were identified annually, which indicate the existence of two generations. The first flight phase was relatively weak. It began in mid June and reached its peak in mid July. It was followed by a much extended flight phase of the second generation from mid August to October with a peak in early September (calendar week 36/37). In addition to the surveillance of the flight, basic data on the sex ratios and the appearance of the two main phenotypes of *C. perspectalis* (white and melanic morph) were obtained. It could be shown that neither the proportions of females nor the amount of melanic moths differ significantly between the two flight phases. The proportion of melanic moths was on average 14% ± 2.9% and differed only slightly over the years. Overall, it can be stated that both trap systems are suitable for the observation of the flight activity and thus also for the proper timing of control measures. The detection of females by using light traps is not necessary for this purpose.

Key words: Box tree moth, light- and pheromone traps, flight activity, melanic morph

Zusammenfassung

Zwischen 2012 und 2015 wurde die saisonale Flugaktivität von Freilandpopulationen des invasiven Buchsbaumzünslers *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) im Rhein-Main-Gebiet in Südhessen erfasst. Der Falterflug wurde an zwei Standorten durch eine Lichtfalle (2012-2014) sowie mehrere Pheromonfallen (2013-2015) aufgezeichnet. Es konnten zwei deutliche Hauptflugzeiten identifiziert werden, die auf zwei Generationen im Jahr schließen lassen. Die erste Flugphase war stets schwach ausgeprägt, begann Mitte Juni und gipfelte Mitte Juli. Es folgte eine sehr umfangreiche und intensive Flugphase der zweiten Generation von Mitte August bis Oktober, mit einem Höhepunkt Anfang September (Kalenderwoche 36/37). Darüber hinaus wurden grundlegende Daten zum Auftreten der Geschlechter sowie der zwei Hauptphänotypen (weiße und

melanisierte Morphe) der angelockten *C. perspectalis* Falter erarbeitet. Weder die Proportion der Geschlechter noch die des Auftretens der braunen Morphe unterschieden sich signifikant im Vergleich der beiden Flugphasen. Mit der Lichtfalle wurde eine wiederkehrende Mehrheit von männlichen Faltern festgestellt und es konnte gezeigt werden, dass weibliche Falter ihren Flug im Frühjahr nicht vor dem der Männchen beginnen. Der Anteil an Faltern der braunen Farbvariante betrug im Durchschnitt 14% ± 2.9% und unterschied sich über die Jahre hinweg nur gering. Insgesamt kann festgehalten werden, dass sich beide Fallensysteme zur Erfassung der Flugaktivität und somit auch zur Terminierung von Bekämpfungsmaßnahmen eignen. Die Aufzeichnung der Weibchen mit Lichtfallen ist dazu nicht zwingend notwendig.

Stichwörter: Buchsbaumzünsler, Licht- und Pheromonfallen, Flugaktivität, braune Morphe

Introduction

Ten years ago, in 2006, the box tree pyralid *Cydalima perspectalis* (Walker 1859) (Lepidoptera: Crambidae) syn. *Glyphodes perspectalis* Guenée, *Palpita perspectalis* Hübner and *Diaphania perspectalis* Hübner (Mally & Nuss 2010) arrived in Europe (Billen 2007; Krüger 2008) and became highly invasive. Until now more than 20 European countries are affected (Nacambo et al. 2014). In Europe, *Buxus* plants are one of the most popular ornamental shrubs with great cultural significance. Most frequently planted species are different varieties of *Buxus sempervirens* L. and *B. microphylla* Sieb. & Zucc.. Thousands of plants in private gardens, cemeteries, public parks and palace grounds are affected by feeding damage of the new pest or already had to be replaced. Besides the cultural and economic effects, potential ecological effects may occur due to the additional threat to the natural *Buxus* stands. *B. sempervirens* L. in Central and Southern Europe, *B. balearica* Lam. in the Mediterranean area (Di Domenico et al. 2012) and *B. colchica* Pojark, in the Caucasus, especially in the forests of Georgia (Matsiakh 2016) are highly endangered. It can be assumed that no *Buxus* species or variant exists, which is not serving as potential host plant for *C. perspectalis* and a lot of the rare natural stands are already concerned.

C. perspectalis is a monophagous pest and its life cycle occurs completely on host plants of the genus *Buxus*. Moths are flying in the late evening and night. After mating and dispersal adult females lay their eggs in batches mostly on the underside of the leaves. Eggs are going to hatch out within ten days. Larvae emerge and undergo a feeding period of several weeks. At the beginning small larvae feed aggregated, causing damage on the exterior leaf layer and form loose nests in the plant. Later

on, they spread and feed solitary on the foliage, typically sparing the vein and often additionally attacking the bark. Six larval instars are usually passed until the pupation. Larvae spin a cocoon among a few leaves and evolve to a pupa from which the moth will grow out and the cycle begins anew. Larvae of the next generation emerge in autumn. They feed until the third larval instar and overwinter as small larvae in a closely spun cocoon between two leaves. In spring, feeding activity restarts. There are different statements to the number of occurring generations in Germany. Albert and Lehneis (2010) assumed at least three generations in Baden-Württemberg, but also two generations were postulated (CABI 2013; Zimmermann 2014). However, there is still a lack of detailed and documented information on the flight phenology.

Figure 6 Trap types. Light trap (left) and pheromone trap, funnel type (right).

It is known that different types of wing color varieties exist in *C. perspectalis* populations. The typical morph is white with a dark brown margin and small characteristically crescent-shaped white marks on it. The body is white with a brownish abdominal segment (Figure 7 A). Body and wings of the melanic morph are almost uniform dark brown with the exception of the two white marks (Figure 7 C), also described by Sáfián and Horváth (2011) and Székely (2011). In addition, an intermediate phenotype exists with an extra brown margin at the forewings (Figure 7 B), also recognized by Pan et al. (2011) in China, but not considered in this investigation. The melanic morph can already be identified at the pupal stage (Figure 7 D).

Monitoring is an important component when dealing with invasive species, according to assess the spread, population levels and the seasonal flight activity per year (Valles et al. 1991). The determination of the adult flight phases per year can lead to conclude the number of completed

generations and additionally the phases of egg deposition and larval feeding. It enables to time diverse control measures adapted to the occurring developmental stage in field. This can reduce dispensable insecticide applications, resulting in a more efficient pest management system. The investigation of the occurrence and the proportion of the sexes and morphological varieties in *C. perspectalis* populations offer information on potential seasonal differences which may lead to a developmental advantage, for instance on overwintering. This could be indicated by an earlier flight of a particular sex or melanic moths. Laurent and Frérot (2007) reported problems of monitoring the European corn borer (*Ostrinia nubilalis* Hübner, Crambidae) with pheromone traps. It remains unclear whether flight curves plotted from pheromone trap captures truly reflect moth phenology because of the earlier flight of the female moths which cannot be lured by sex pheromone trapping. De Jong et al. (1996) postulated that the melanic morph of the ladybird (*Adalia bipunctata* Linnaeus, Coccinellidae) exhibits higher body temperature, tending to warm up slightly faster than the non-melanic ones. Morphs appeared to be the principal factor influencing activity.

The emphasis of this study was to observe *C. perspectalis* populations for consecutive years with two monitoring systems, pheromone and light trapping (Figure 6). We wanted to find out exact information on the seasonal flight activity patterns and whether there are differences in the temporal flight sequence of the sexes and of the two major morphological varieties (Figure 7).

Figure 7 Morphological varieties and sex dimorphism of *C. perspectalis*. (A) moth; typical white morph, (B) moth; intermediate morph, (C) moth; melanic morph, (D) pupae; typical white (above) and melanic morph (below), (E) Male, hair-pencil present on the tip of the abdomen (left) Female, abdomen without hair-pencil (right).

Material and methods

The study was conducted at two sites in the Rhine-Main region nearby Frankfurt in South Hessia, Germany. The "Convent Garden Seligenstadt" (50°2'38.75"N 8°58'31.25"E) has an area of about 3 ha and contains more than 3.5 km *Buxus* hedges as bed enclosures. The historical "Old Cemetery Darmstadt" (49°51'53"N 8°40'6"E) has a ground area of 13.5 ha and includes numerous individual *Buxus* plants or groups on graves and the enclosure of the irrigation systems. On both sites, infection with *C. perspectalis* was high and no insecticide treatment occurred, except of *Bacillus thuringiensis kurstaki* applications at Seligenstadt in 2013 and 2014.

The *light trap* (Figure 6) was installed for three consecutive years (2012, 2013 and 2014) in the herb garden of the cloister Seligenstadt, South Hessia, which has an area of 600 m². It was purchased from the company Fiebig-Lehrmittel (Berlin, Germany). The total height of the trap was 1.3 m. A square case (length 51 x width 51 x height 40 cm) was forming the standing base of the trap. In the middle of this main chamber, a plastic catch tray (l 35 x w 29 x h 18 cm) was placed with a small hole below the funnel (diameter 26 cm, 7 cm at the tail). Above the funnel, the baffle (h 70 cm) and the light sources (two NARVA Colourlux plus bulbs) was rising out of the case, protected by a plate (d 52 cm) on top against rain. Moth flight was surveyed from May/ June until September/ October (Table 3). Trap was daily checked and the content was examined. The number of caught moths, sex and the morphological variety of the wing color (white and brown) was determined. Sex was assessed by recognizing abdominal dimorphism. Males could be identified on their hair-pencil at the last abdominal segment (Figure 7 E).

Pheromone trapping was conducted between 2013 and 2015 in Darmstadt and Seligenstadt, to survey the seasonal moth flight activity. Investigations on the pheromone compositions, lures and traps were already carried out in 2013 and 2014 in cooperation with the company Pherobank B.V. (NL). Thus, at the end of 2014 a combination of a reliable pheromone composition and a valuable lure was discovered for optimal capturing of *C. perspectalis* (data will be published elsewhere). Funnel traps (Figure 6) proved to be more suitable on moth trapping than the delta trap type and were appropriate for an effective monitoring (unpublished data). Pheromone lures were based on the two main active components (Z)- and (E)-11-hexadecenal (Kawazu et al. 2007; Kim & Park 2013) and offered by polyethylene vials. Lures were exchanged after 3-4 weeks. In our trials, pheromone traps (d 16.5 cm, h 21.5 cm) were set up in approximately 1.6 m height on trees or shrubs with the greatest possible distance to each other (about 25 m in Seligenstadt and more than 100 m in Darmstadt). 8-12 traps were used per comparison phase and site. They were assessed weekly and

the local position was randomized. In 2015, observation could only be conducted in Darmstadt. In Seligenstadt the experiment had to be stopped because of repeated vandalism on the pheromone traps.

Statistics were done with RStudio (Version 0.99.489 – © 2009-2015 RStudio, Inc.; R Version 3.2.2). To analyze the count data of caught moths (distribution of sex and morphological variety) within one year, Chi-squared tests for given probabilities were used. To compare mean proportions [%] 2-sample tests for equality of proportions with continuity correction were done.

Results

Seasonal flight activity

In general, moth catch was summarized to calendar weeks (cw) and could be observed from June (rarely May/ cw 22) until October (cw 42). Two main flight periods were clearly identified with both trapping systems. The first flight phase arose from mid June (cw 24) until late July (cw 29). At this time, the adults of the overwintering generation emerged. Catch decreased at late July/ early August (cw 31/32) in both trapping systems. Then a much extended second flight phase occurred from mid August (cw 33) until October (cw 42) where the major flight was performed with a peak in September (cw 36) (Figures 8 and 9). Thus, according to these results two main flight periods were identified annually. In 2014, a decreased number of trapped moths were documented for pheromone trapping in Seligenstadt, resulted from repeated vandalism on the traps. But also for light trapping a lower population was recorded, probably due to the *Bacillus thuringiensis* applications conducted in 2013 and 2014.

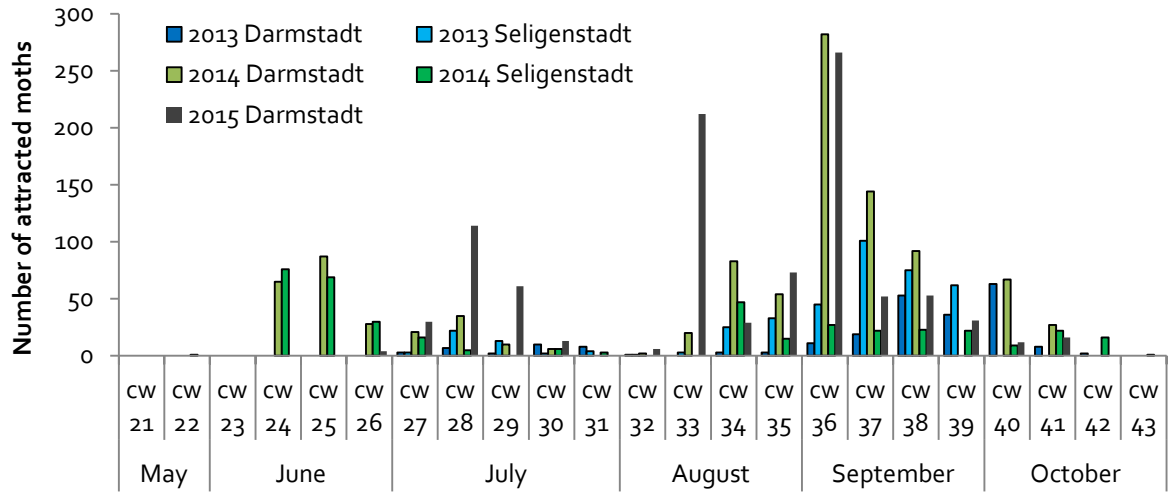


Figure 8 Seasonal flight activity detected by pheromone trapping in the years 2013–2015 at Seligenstadt and Darmstadt (cw = calendar week).

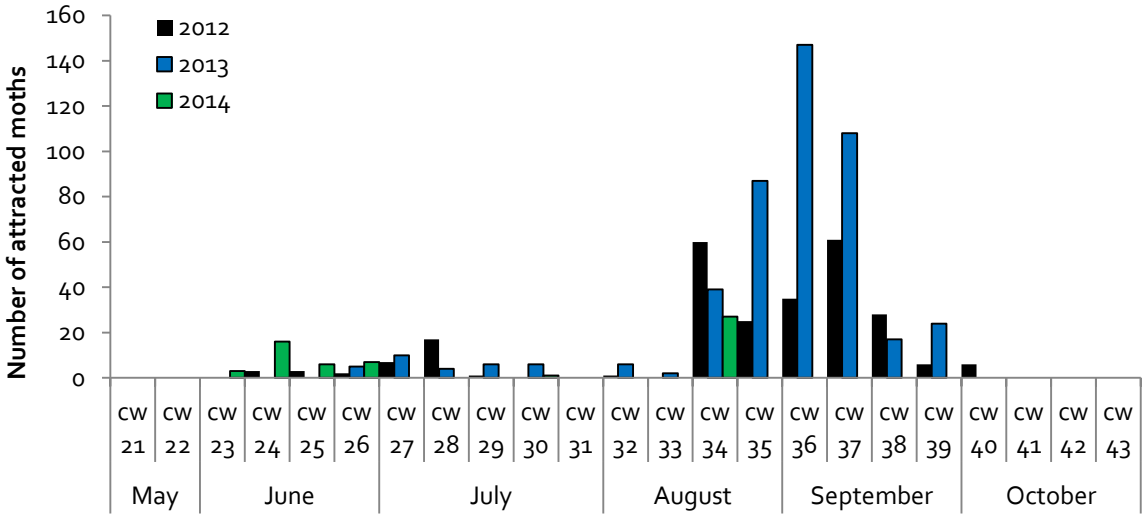


Figure 9 Seasonal flight activity detected by one light trap in the years 2012–2014 at Seligenstadt (cw = calendar week).

First catch

In 2012, only the light trap in Seligenstadt was installed and the first catch (Table 3) was registered in early June (cw 24). In 2013, the pheromone trapping started and the first moth catch occurred in late June/ early July (cw 27) for both sites. First catch of the light trap was shown one week earlier (cw 26), but the trap installation was realized one week earlier as well. In 2014, a single male was caught in a pheromone trap in Seligenstadt very early, in May (cw 22). Other first catch in 2014 were observed in early June (cw 23) caught by the light trap and one week later in cw 24 caught by a pheromone trap in Darmstadt, which was not installed before cw 23. There was no major difference between the temporal recordings of the first catch in the two trap systems. First females were

caught together with first males in the light trap but in less number. Early captured moths were mostly white and a potential earlier flight of the melanic moths in spring was not documented.

Table 3 First catch of *C. perspectalis* during the observation of seasonal flight activity (2012-2015) by light and pheromone trapping at the two study sites (Seligenstadt = SE and Darmstadt = DA).

Trap type and site	Trap installation period	First catch*	Number and sex	Morph
Light trap (SE)	24.05.-09.10.2012	cw 24	1 male, 2 females	white
Light trap (SE)	18.06.-10.10.2013	cw 26	4 males, 1 female	white
Light trap (SE)	22.05.-19.10.2014	cw 23	2 males, 1 female	white
Pheromone traps (SE)	25.06.-03.10.2013	cw 27	3 males	white
Pheromone traps (SE)	22.05.-23.10.2014	cw 22	1 male	white
Pheromone traps (DA)	25.06.-16.10.2013	cw 27	3 males	white
Pheromone traps (DA)	05.06.-16.10.2014	cw 24	60 males, 5 males	white, brown
Pheromone traps (DA)	19.06.-30.10.2015	cw 25	4 males	white

*cw = calendar week

Sex distribution

Light trapping enabled to record the sex distribution of caught moths. In total, sex could be determined for a number of 743 moths. For a small amount of moths (4%) determination was not possible, because catch were partially soaked by rain and/or the abdomen was damaged. To examine the occurrence of possible temporal differences in the sex distribution, catch was analyzed separately for the two flight phases per year (first: June-July and second: August-October). Mean distribution (2012-2014) of the first flight phase was 54% ± 17% males and 46% ± 17% females, whereas we found 60% ± 3.8% males and 40% ± 3.8% females in the second flight phase (Table 4). There is no significant difference in the proportions of sex in the two flight phases ($\chi^2 = 0.51$, df = 1, p = 0.4751). First females were recorded together with first males in the light trap (Table 3).

However, the statistical analyses of observed moth catch per year showed a significant surplus of caught males during the first flight phase in 2014 ($\chi^2 = 4.84$, df = 1, p = 0.03), the second flight phase in 2012 ($\chi^2 = 4.74$, df = 1, p = 0.03) and in 2013 ($\chi^2 = 9.94$, df = 1, p = 0.002). Sex ratios (males: females) ranged between 0.6 and 2.6 (Table 4). Similar ratios were found if considering only the brown variety; ranging between 1.3 and 1.7. Only in 2012 a surplus of females could be detected for the first flight phase (sex ratio 0.6), but the difference was not significant ($\chi^2 = 2$, df = 1, p = 0.1573). There is no evidence that *C. perspectalis* females begin their flight earlier than males.

Table 4 Occurrence of sex (males and females) and morphological varieties (white and brown) of *C. perspectalis* (proportion and number) during the two flight phases (first: June-July and second: August-October) detected by light trapping (2012-2014). Asterisks indicate statistical significance[a].

year	first flight phase				second flight phase			
	males	females	sex ratio	χ^2	males	females	sex ratio	χ^2
2012	38 (12)	62 (20)	0.6	2.0	57 (124)	43 (92)	1.3	4.7*
2013	53 (16)	47 (14)	1.1	0.1	58 (238)	42 (174)	1.4	9.9*
2014	72 (18)	28 (7)	2.6	4.8*	64 (18)	36 (10)	1.8	2.3
mean ± sd	**54 ± 17**	**46 ± 17**			**60 ± 3.8**	**40 ± 3.8**		
	white	brown		χ^2	white	brown		χ^2
2012	91 (30)	9 (3)		22.1*	88 (194)	12 (27)		126.2*
2013	81 (26)	19 (6)		12.5*	82 (354)	18 (76)		179.7*
2014	81 (26)	19 (6)		12.5*	75 (21)	25 (7)		7.0*
mean ± sd	**84 ± 5.8**	**16 ± 5.8**			**82 ± 6.6**	**18 ± 6.6**		

[a] χ^2- test for given probabilities ($\chi^2 > 3.8$, df = 1, $p < 0.03$)

Morphological variety

Early caught moths were mostly white and an earlier flight of the brown morph in spring became not apparent (Table 3). The proportions of the brown moth variety per month summarized per trap system (and site within pheromone trapping) ranged between 3% and 27%. Using pheromone traps, a mean proportion of 84% white to 16% ± 0.1% brown lured male moths per year was observed in Seligenstadt and 89% white and 11% ± 3.4% brown moths in Darmstadt over the years of the study. Light trapping detected a similar ratio of 84% white to 16% ± 1.9% brown moths in Seligenstadt. Thus, on average, a proportion of 14% ± 2.9% melanic moths per year could be expected (Figure 10).

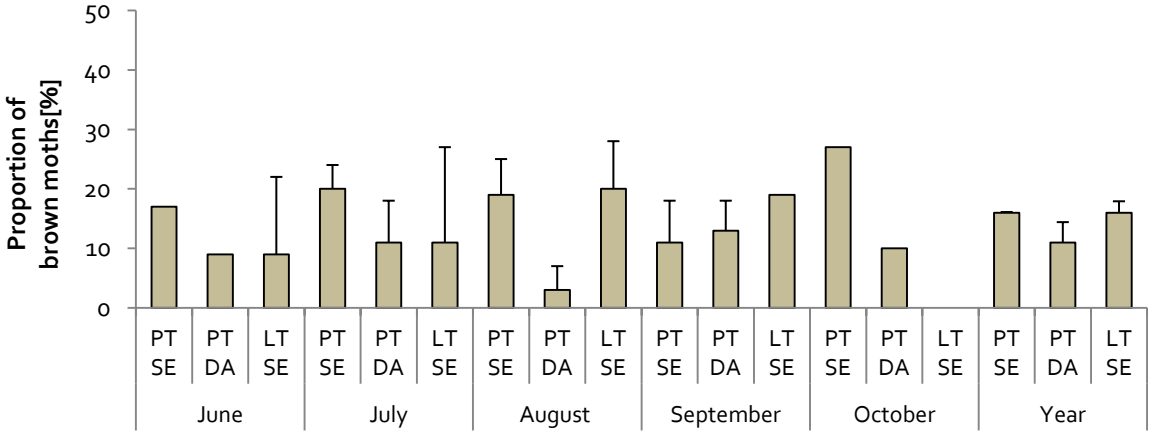


Figure 10 Mean proportion [%] of the brown variety of *C. perspectalis* moths per month. Caught moths (2013 and 2014) were summarized per trap system (Pheromone trap= PT and Light trap= LT) and study site (Seligenstadt= SE and Darmstadt= DA).

In addition, the proportion of caught melanic moths per flight phase evaluated per *light trapping* displayed similar range. Mean distribution (2012-2014) of the first flight phase was 84% ± 5.8% white and 16% ± 5.8% brown colored moths. In the second flight phase 18% ± 6.6% brown *C. perspectalis* could be observed. There is no significant difference in the proportions at the two flight phases (χ^2 = 0.035436, df = 1, p = 0.8507) (Table 4). Mean percentage of melanic moths of the first flight phase detected by *pheromone trapping* in *Seligenstadt* (2013 and 2014) was 20% ± 4.2%. In the second flight phase 12% ± 4.9% brown moths could be observed. However, the difference in the proportions of the morphological varieties of the two flight phases is not significant (χ^2 = 1.8229, df = 1, p = 0.177) (Table 5). In *Darmstadt* a percentage of 13% ± 5.7% brown moths could be determined for the first flight phase and 11% ± 2.8% for the second flight. There is no significant difference in the proportions of the morphological varieties of the two flight phases (χ^2 = 0.047348, df = 1, p = 0.8277) (Table 5), so there is no incidence for earlier flight of the melanic moths in spring and no significant surplus of melanic moths observed in the first flight phase.

Table 5 Occurrence of morphological varieties (white and brown) of *C. perspectalis* (proportion and number) during the two flight phases (first: June-July and second: August-October) detected by pheromone trapping (2013-2014) at the two study sites (SE = Seligenstadt, DA = Darmstadt). Asterisks indicate statistical significance[a].

site/ year	**first flight phase**			**second flight phase**		
	white	brown	χ^2	white	brown	χ^2
SE/ 2013	77 (34)	23 (10)	13.1*	84 (291)	16 (54)	162.8*
SE/ 2014	83 (170)	17 (35)	88.9*	91 (316)	9 (33)	229.5*
mean ± sd	**80 ± 4.2**	**20 ± 4.2**		**88 ± 4.9**	**12 ± 4.9**	
DA/ 2013	83 (25)	17 (5)	13.3*	87 (173)	13 (26)	108.6*
DA/ 2014	91 (230)	9 (22)	108.6*	91 (704)	9 (67)	526.3*
mean ± sd	**87 ± 5.7**	**13 ± 5.7**		**89 ± 2.8**	**11 ± 2.8**	

[a] χ^2 - test for given probabilities ($\chi^2 > 3.8$, df = 1, $p < 0.0005$)

Discussion

It is well known, that reliable monitoring is based on a specific, effective and easy applicable trapping system. Pheromone trapping enables a selective observation of a target organism and can be provide high catch if the right pheromone is used. One major drawback might be that only males can be lured. Laurent and Frérot (2007) reported problems of monitoring the European corn borer (*Ostrinia nubilalis* Hübner, Crambidae) with pheromone traps. It remained unclear whether flight curves plotted from pheromone trap captures truly reflect moth phenology because of the earlier

flight of the *Ostrinia* female moths which cannot be lured by sex pheromone trapping. Additionally blends lose their attractiveness or attract different species if the pheromone components are not combined in the exact suitable proportions. When using light traps there is the major advantage, that both sexes are attracted. This is indispensable to acquire data on biological characteristics, like the timely occurrence and proportions of both sexes in field. Drawbacks are the dependence on electricity, the bulkiness and the attraction of different non-target and beneficial insects. Also maintenance effort is very high.

This study provides new information about the moth flight activity of *C. perspectalis* per year and the usability of different trap types to monitor the pest. Evaluations with light and funnel traps at two sites in the Rhine-Main region showed two clearly separated flight phases with both trap types (Figure 8 and Figure 9). The highest flight activity of this invasive pest occurs in mid July and early September. That agrees with the observed seasonal development of *C. perspectalis* postulated by Zimmermann (2014) and by the CABI (2013) for South Germany. Data on the flight phenology, including the number of generations a year, have also been published for different populations in Asia and Europe (Table 6).

Table 6 Number of *C. perspectalis* generations per year for different countries and regions.

no. of generations	country	region	reference
up to five	China	Zhejiang province	SHE AND FENG (2006)
up to four	China	Youxi Fujian	WANG (2008)
three	China	Shanghai	TANG et al. (1990)
three	China	Xian	CHEN et al. (1993)
three	Japan	Tokyo	MARUYAMA AND SHINKAJI (1987)
three	Italy	Verona	SANTI et al. (2015)
two	Korea	Seoul	PARK (2008)
two	Russia	Sochi	KARPUN AND IGNATOVA (2013)
two	Switzerland	Northern	NACAMBO et al. (2013)
up to four	Switzerland	Southern	KENIS et al. (2013)
two	Croatia	Varaždin	MATOŠEVIĆ (2013)
three	Switzerland	Basel	LEUTHARDT et al. (2010)

Three to five generations were described for eleven different Chinese populations depending on the climatic conditions of the particular regions (Wan et al. 2014). Wang (2008) showed emergence periods of the first, second, third and fourth generation starting on the middle ten days of May, the first ten days of July, the last decade of September and the middle and last ten days of November,

respectively. Serious damage has been observed in China from May to September. In Japan, three generations were described for a Tokyo population where adults appeared from mid-May to late June, from late July to late August and from late August to mid-September (Maruyama & Shinkaji 1987). In Korea two generations were observed, first from early June to late June and second from mid- August to early September (Park I. 2008), similar to the pattern that we found here. In Russia, also two generations were recognized. The first generation remained almost unnoticed, flight of the second generation was observed in the first ten-day period of September (Karpun & Ignatova 2013). In Europe, two to four generations occur. Three generations are published for Italy (Santi et al. 2015) and the region of Basel in Switzerland (Leuthardt et al. 2010). In warmer areas of Switzerland even four generations per year may occur (Kenis et al. 2013). Populations in northern Switzerland (Nacambo et al. 2014) and Croatia (Matošević 2013) were monitored and two generations per year were described. In Georgia, the exact number of generations could not be defined until now but the flight period was observed in summertime (late July) as well as in late October (Matsiakh 2016).

This study also gives first results concerning abundance of the melanic moths and proportions of sex in field populations. There are many insect orders and moth species with natural incidence of melanism, i.e. the occurrence of morphological variants that are mostly or completely dark in pigmentation. Common lepidopteran examples are the Peppered moths (*Biston betularia* Linnaeus, Geometridae) from the UK and the tiger swallowtails (*Papilio glaucus* Linnaeus, Papilionidae) from Florida (True 2003). In recent decades a wide range of explanations on the cause of melanic morphs and the effect on fitness have been suggested and discussed. The attention was mainly focussed on the genetic origin and inheritance in connection with changing environmental conditions as well as predation. For example Cook & Saccheri (2013) reviewed the ideas about industrial melanism and its evolutionary changes in peppered moth populations, because in some cases melanic mutants became dominant. Liu et al. (2015) reported on the association of beet armyworm (*Spodoptera exigua* Hübner, Noctuidae) pupal melanism and fitness heightening. Our results detect no dominance of the melanic moths in the trapped populations; proportions were nearly constant for both trap types, in every year, for both flight phases and at both sites and ranged between 11% and 20% (Table 4 and Table 5). The mean proportion of melanic moths per year was 14% ± 2.9%. Separated per month, mean proportions (2013 and 2014) had a wider range (between 3% and 27%) but there was no indication on a raised amount of melanic moths in spring after overwintering or in one of the two flight phases.

In most lepidopteran species the appearance of males occurs earlier in the season than of females of the same species (protandry) (Wiklund & Fagerström 1977; Iwasa et al. 1983; Zonneveld

1992).Wiklund and Fagerström (1977) advanced hypotheses which explain the incidence of protandry, indicating that males emerge before females to maximize reproductive success. Early occurring males seem to stand a greater chance of mating with one or several virgin females than do males emerging later, maintaining females mate only once (monandry), whereas males are capable of multiple matings (polyandry). But earlier emergence of females (protogyny) additionally occurs among lepidopteran species, for example in the mating system of the diamondback moth (*Plutella xylostella* Linnaeus, Yponomeutidae)(Uematsu & Morikawa 1997). Thus, it is being also part of the complex matter of sexual selection and mating systems of individual lepidopteran species. In our study, one aim was to examine whether *C. perspectalis* females may do begin their flight earlier than males and furthermore to assess if there are differences in the temporal flight sequence of the sexes. First females were recorded together with first males in the light trap and the mean proportion (2012-2014) of females during the first flight phase was 46% ± 17% and 40% ± 3.8% during the second. Thus, we can assume that obviously no protogyny occurs and flight curves plotted from pheromone trap captures truly reflect the exact moth flight of both sexes.

The present findings concerning the use of light and pheromone traps clarified the usability of pheromone traps to survey the seasonal flight activity of *C. perspectalis*. It can be stated that both trap systems are suitable for exact observation. But the elevated sex ratio in field populations pointed out that an inclusion of the females by using light traps is not necessary. Results lead to conclude two completed generations per year and additionally enables the early detection of egg deposition and larval feeding phases. Thus, diverse control measures can be adapted to the occurring developmental stage in field, for example to release egg parasitoids like *Trichogramma* wasps (Trichogrammatidae) during the time of egg deposition or to time insecticides precisely that are directed to first larval instars. This can reduce dispensable insecticide applications, resulting in a more efficient pest management system. Additionally, the documentation of the regular occurrence of melanic moths provided useful insights into the biology of this invasive organism.

Chapter II. GÖTTIG S. & HERZ A. (2016) *Biocontrol Science and Technology*, 26 (11) 1471-1488.

Are egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) promising biological control agents for regulating the invasive Box tree pyralid, *Cydalima perspectalis* (Lepidoptera: Crambidae)?

Abstract

The Box tree pyralid, *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) is an invasive species in Europe, not yet regulated by natural enemies. It is highly destructive to ornamental and native *Buxus* due to defoliation by larval feeding. The ability of eight *Trichogramma* species (*T. brassicae* Bezdenko, *T. bourarachae* Pintureau & Babault, *T. cacoeciae* Marchal, *T. cordubensis* Vargas & Cabello, *T. dendrolimi* Matsumura, *T. evanescens* Westwood, *T. nerudai* Pintureau & Gerding and *T. pintoi* Voegele) to parasitize and locate eggs of *C. perspectalis* was assessed in the laboratory. In direct observation tests, all species started to examine and oviposit into exposed eggs. Resulting egg parasitism was low and ranged between 4% and 20% for most of the parasitoid species after three days of exposure. Only *T. dendrolimi* parasitized significantly more host eggs with parasitism rates above 40%. On average, two to three progeny emerged from one host egg and the resulting sex ratio was female-biased, indicating suitability of the target host for progeny production. In subsequent cage tests on potted *Buxus* plants, again *T. dendrolimi* was the most efficient species in locating host eggs on foliage. We conclude that *C. perspectalis* eggs are attractive to various *Trichogramma* species. *T. pintoi* had the highest number of egg encounters and active females. *T. dendrolimi* females were most able to locate host eggs and to oviposit and produce successfully on them. Nonetheless, further study is needed to clarify the suspected role of potentially egg defensive mechanisms that may impede higher parasitization in *C. perspectalis*.

Keywords: Box tree moth, *Buxus*, host acceptance, host suitability, host location, egg parasitism

Introduction

The Box tree pyralid, *Cydalima perspectalis* (Walker 1859) (Lepidoptera, Crambidae) is a herbivorous insect that is specialized on the single plant genus *Buxus* sp. (van der Straten & Muus 2010; Leuthardt & Baur 2013; Wan et al. 2014). Oviposition, larval development, pupation and overwintering take place on the host plant. The species is native to India, China, Korea, Japan and the Russian Far East (Mally & Nuss 2010) and was recently introduced with infested *Buxus* plants from China into Europe (Billen 2007; Leuthardt et al. 2010). Adults and larvae were first detected in 2007 in Southern Germany, in the vicinity of the port of trans-shipment in Weil am Rhein, and next in Switzerland (Billen 2007; Krüger 2008). Until now, 16 European countries have been invaded by *C. perspectalis*. According to a Swiss study, the repeated supply of infested *Buxus* plants in garden centers in the surroundings of Basel contributed to the fast spread of *C. perspectalis* (Leuthardt et al. 2010). Based upon a climate model, Nacambo et al. (2014) suggested that *C. perspectalis* would probably continue its spread across all of Europe, except for Northern Fenno-Scandinavia, Northern Scotland and high mountain regions.

Due to the development of several generations per year, *C. perspectalis* populations can reach high-density levels leading to complete defoliation of host plants. Since its invasion in Europe, outbreaks occurred on ornamental box trees in nurseries, cemeteries, historical monuments, public parks and gardens as well as on native *Buxus* plants in forests. One of the rare native *Buxus* forests in Germany was completely destroyed by this pest combined with an infestation by the fungus *Cylindrocladium buxicola* Henricot (John & Schumacher 2013). There are also natural areas of *Buxus* plants, mainly as understorey of broadleaf forests or woodlands, garrigues and calcareous grasslands in the Pyrenees and Southern France. More isolated stands occur around the Jura Mountains, in Central France and the southern European Peninsulas as well as minor stands located in Britain, Belgium, Luxembourg, Germany, Sardinia, Montenegro and Kosovo (Di Domenico et al. 2012). All these stands are natural habitat types, which demand classification as special areas of conservation and are now seriously endangered due to the arrival of this destructive pest.

The use of insecticides on ornamental and natural box plants is often disliked or even prohibited, because ornamental *Buxus* plants in Europe are frequently part of private and public gardens as well as cemeteries and historical parks, where specific permissions must be complied with. In addition, large-scale application of broad-spectrum insecticides in nature-reserves may cause unwanted side effects on non-target organisms like endangered lepidopteran species. Current pest management in its native range of Japan and Asia relies mainly on chemical insecticides, such as pyrethroids (Wan et

al. 2014). According to the European Academies Science Advisory Council (EASAC) frequent applications of pyrethroids and neonicotinoids may endanger non-target organisms. Widespread use of neonicotinoids cause severe negative effects on organisms that provide ecosystem services including pollination and natural pest control (EASAC 2015). The use of plant protection products based on *Bacillus thuringiensis* is currently the only alternative and available effective biological control method. Larvae of *C. perspectalis* are highly susceptible to the strains *B. t. kurstaki* and *B. t. aizawai* and even older instars may die at high rates after ingestion of sprayed foliage (unpublished data). However, these preparations need to be sprayed at least twice against each generation and treatments require a permission and proper surveillance of this multivoltine species. If not applied appropriately or timely, damage by larval feeding continues. Although Rose et al. (2013) found the baculovirus *Anagrapha falcifera nucleopolyhedrovirus* (AnfaNPV) to be infective for *C. perspectalis* in a recent laboratory study; no plant protection product was subsequently developed. Consequently, the demand for more environmentally and user friendly, effective control methods is urgent. In Europe, China and Japan, some common natural enemies were described but they are reported as being polyphagous and their impact on *C. perspectalis* populations remains low and mostly unknown (Wan et al. 2014). No specialized natural enemy was found yet and initial investigations were not very promising. Only one more specific and well adapted egg-larval parasitoid, *Chelonus tabonus* Sonan (Braconidae) was found in China (Wan et al. 2014) but first experiences using the commercially available larval parasitoids *Bracon brevicornis* Wesmael and *Bracon hebetor* Say (Braconidae) argued that these parasitoids could not complete their development in this new host (Zimmermann & Wührer 2010).

This study was addressed to find an environmentally and user friendly, effective control method, which is commercially available and do not require permitting or testing for introduction. Especially egg parasitoids prevent hatching of larvae, thus reducing the risk for defoliation and have not to deal with the large amounts of dibasic alkaloids in *C. perspectalis* larvae. On these grounds, *Trichogramma* wasps may be a suitable tool for biological control of the Box tree pyralid.

Idiobiont egg parasitoids of the genus *Trichogramma* Westwood comprise more than 200 species worldwide (Querino et al. 2010) and are known to parasitize several lepidopteran pests, thus preventing any feeding damage (Mills 2010). Research activities on *Trichogramma* as biological control agent have a long tradition of more than 100 years. In the 1970s, mass rearing and releases began and became commercial business (Smith 1996). Application of *Trichogramma* is now a very important part of integrated pest management worldwide. Wasps belong to the most successfully applied biological control agents in inundative releases, even at large scale like in corn or cotton

(Mills 2010). In Germany, the biological control of the European Corn Borer, *Ostrinia nubilalis* Hübner with *Trichogramma brassicae* Bezdenko is well-established since more than 25 years and nowadays the commercial application exceeds more than 22000 ha (Jehle et al. 2014).

Trichogramma is considered to be rather generalistic in comparison to other parasitoids but no *Trichogramma* species was found associated with *C. perspectalis* in its native range (Wan et al. 2014). It is known that close relatives of the lepidopteran family Crambidae, e.g. *Diaphania hyalinata Linnaeus* (Melo et al. 2007), *Diatraea saccharalis* Fabricius (Forti Broglio-Micheletti et al. 2007), *Ostrinia nubilalis* Hübner (Voegele 1981), *Palpita unionalis* Hübner (Herz & Hassan 2006), and Pyralidae, e.g. *Ephestia cautella* Walker (Steidle et al. 2001), *Plodia interpunctella* Hübner (Grieshop et al. 2006) are accepted as hosts by several *Trichogramma* species. Therefore it may be promising to explore their general ability to attack and parasitize also this new host species. According to Vinson (1976) and Hassan (1993) evaluation of host acceptance and host suitability are important criteria when selecting *Trichogramma* species for biological control. Also their ability to locate the host on the plant (host location) needs to be determined in order to predict the prospect for successful releases in practice. Taking these criteria into account in a sequential approach (Herz & Hassan 2006), eight species of the genus *Trichogramma* were tested in this laboratory study for their potential capacity to exploit *C. perspectalis* as a host. The study is considered as first step to identify suitable biological control agents which may be used in inundative releases against this new invasive pest in Europe.

Material

Trichogramma wasps

Four commercially available *Trichogramma* species (*T. brassicae, T. cacoeciae, T. dendrolimi* and *T. evanescens*), considered to be native in Germany as well as four other species occurring mainly in Southern Europe (*T. bourarachae, T. cordubensis*, *T. nerudai* and *T. pintoi*) were investigated (Table 7). All species were maintained in our rearing collection. *Trichogramma* wasps were reared in glass vials on the eggs of the Angoumois grain moth *Sitotroga cerealella* Olivier, fixed with non-toxic glue (3 g Tragant/50 ml water) on card stripes. Parasitization and subsequent incubation of these parasitized egg cards followed a standard rearing procedure under fluctuating temperature conditions at 26 °C, 70% RH, 18 °C, 90% RH, 16 h light: 8 h dark (Hassan 1993). Freshly hatched parasitoids (females ≤ 24 h old, fed and mated) were used for the experiments.

Table 7 *Trichogramma* species which were evaluated as potential biological control agents against *C. perspectalis* in the present study. Year of starting the rearing culture, the original host and the country of origin are listed. Abbreviations (Label) for species name are used in the following tables and figures.

***Trichogramma* species**	**Label**	**Reared since**	**Host/crop system**	**Origin of strain**
T. brassicae Bezdenko	BRA	1993	*Ostrinia nubilalis*	Moldavia[1]
T. cacoeciae Marchal	CAC	1990	*Cydia pomonella*	Germany
T. dendrolimi Matsumura	DEN	1990	*Cydia* (baits)*, apple	Germany
T. evanescens Westwood	EVA	2006	*Pieris* sp. in cabbage	Germany
T. bourarachae Pintureau & Babault	BOU	2002	*Sitotroga* (baits)*, olive	Egypt[2]
T. cordubensis Vagas & Cabello	COR	1993	*Helicoverpa armigera*	Portugal[3]
T. nerudai Pintureau & Gerding	NER	2002	*Sitotroga* (baits)*, olive	Portugal[2]
T. pintoi Voegele	PIN	2010	*Helicoverpa armigera*	Uzbekistan

*(baits) = eggs of reared hosts were exposed in the crop to bait egg parasitoids.
[1] collected 1975 in Moldavia, since 1993 in mass rearing production in Germany.
[2] Herz et al. (2007)
[3] Silva et al. (1999)

C. perspectalis egg masses

C. perspectalis was reared under laboratory conditions since 2010. Larvae were fed on watered box tree twigs (*Buxus sempervirens* var. arborescens *Linnaeus*) in transparent Perspex cylinders (40 cm height x 19.5 cm diameter) with textile covers. Adult moths were kept in rearing cages (length 40 cm x width 36 cm x height 50 cm) with diluted honey (5%). Short box tree twigs (15-20 cm) were regularly introduced into the cages for egg deposition. Single leaves with one *C. perspectalis* egg mass (≤ 24 h old) each collected from the box tree twigs for the experiments. Females of *C. perspectalis* had deposited the egg masses during the preceding night.

Buxus plants

Plant material (*Buxus sempervirens* var. arborescens *Linnaeus*) was obtained from a box tree nursery (Anzuchtbaumschule Atrops, North Rhine-Westphalia, Germany). For rearing and the host acceptance tests, cut twigs (30 cm) and single leaves were used. For host location experiments potted *Buxus* plants (40-50 cm) were utilized.

Methods

Host acceptance

Host acceptance of eight *Trichogramma* species (Table 7) was explored. To avoid experimental variation due to potential host age discrimination, only organisms of an age less than 24 h were used for the bioassays. One bioassay included 30 replicates. One replicate consisted of one individually caged parasitoid female in a small transparent cube (2 x 2 x 2 cm) containing a single box tree leaf with an host egg mass and a drop of honey agar (Hassan 2000). Mean number of eggs per egg mass was 9.1 ± 5.2. A standardization of the number of eggs per leaf was impossible, since removing eggs from a clustered egg mass or any other manipulation of the egg mass may cause variation in the chemical cues of the host eggs, which has to be avoided. Chemical substances appear to play a major role at almost every level of the host selection process (Vinson 1976).

First, for the selection of females, wasps were confined individually in small glass tubes (7.5 cm x 1 cm diameter) and sexed according to their antennal morphology (Romani et al. 2010) by microscopic examination. The selected females were singly transferred into the experimental cubes, in which one *Buxus* leaf with one egg mass had been positioned. Following the procedure of the quality control guidelines of the International Organisation for Biological Control (IOBC) for evaluating host parasitism by mass-reared egg parasitoids (van Dijken et al. 1986; Hassan 1989; van Lenteren et al. 2003) the behavior of *Trichogramma* females were observed during a four hour observation period in which encounters with an egg (sitting on, drumming on the egg mass or ovipositing) were recorded every 30 minutes (Figure 11). Hence, the maximum possible number of observed egg encounters per female was eight. If an encounter was seen, the behavior of the wasp was observed in more detail with a binocular microscope. After the 4 h observation period, females and egg masses were incubated for 72 h (25 °C, 60% RH, 16 h light: 8 h dark) to ensure sufficient time for parasitation. Afterwards females were removed and egg masses were separately incubated for 7-14 days until the parasitized eggs turned black and parasitoid progeny completed emergence (Figure 11). Emerged *C. perspectalis* larvae from unparasitized eggs were frequently removed. Ten additional egg masses, placed in cubes without adding a parasitoid, served as a control to prove the vitality of the offered eggs used for the experiment. The bioassays took place at 25 ± 2 °C and 60% RH during daytime and were performed four times for each species.

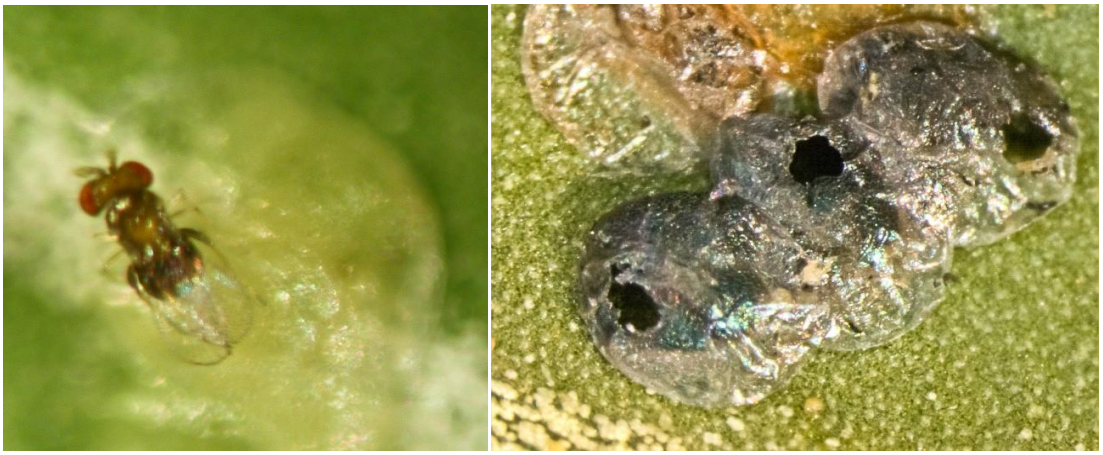


Figure 11 *Trichogramma* female on a *C. perspectalis* egg, evaluating host egg quality before parasitization during the acceptance test (left); box tree leaf with empty *C. perspectalis* host eggs after successful parasitization (eggs turned black) and host embryo killing by a *Trichogramma* wasp. Holes show successful hatching of the *Trichogramma* progeny (right).

Two female-based parameters were determined to assess the host acceptance and the success of parasitism of the individual female: (1) the number of host egg encounters/female and (2) the number of parasitized eggs/female. Calculation of the parasitism rate, i.e. the percentage of parasitized eggs per female, was not appropriate because the number of host eggs varied. Therefore the parasitism rate and further parameters were determined on the level of the cohort (bioassay): (3) the percentage of active females/bioassay during the 4 h observation period, i.e. females with at least one egg encounter/bioassay (4) the percentage of parasitized eggs/bioassay after 72 h incubation, (5) the host cluster acceptance rate (HCAR), i.e. the percentage of parasitized egg masses with at least one parasitized egg/bioassay, and (6) the number of completely parasitized egg masses/bioassay. Based on these six parameters, a ranking was established to compare the acceptance efficacy of the different tested species. Results were ranked from highest to lowest value (1-8) within the determined parameters. According to the average rank for every tested species, the two most effective species against *C. perspectalis* were selected for further testing (Table 8 and Table 9).

Host suitability

To evaluate the suitability of the host eggs for *Trichogramma* females, production of progeny was measured. It is well known that ovipositing females can adjust number and sex of their progeny according to perceived host egg quality (Godfray 1994, King 1987). Therefore (1) the emergence rate and (2) the sex ratio (males: females) of the F1-progeny of each tested *Trichogramma* species were calculated by counting and sexing the emerged wasps per successfully parasitized host egg.

Host location

The two most successful species selected from the host acceptance test were further assessed for their ability to locate the host eggs on the plant. Therefore, cage experiments were conducted with potted *Buxus* plants (*B. sempervirens* var. arborescens) at 25 °C, 60% RH in the laboratory. Each experimental run included three replicates (= cages) per *Trichogramma* species and was performed three times (n= 9).

For each cage (65 cm x width 50 cm x height 100 cm), ten leaves with egg masses (≤ 24 h old) were distributed on one potted *Buxus* plant. The mean number of eggs per offered egg mass was 14 ± 7.5. The leaves with egg masses were carefully fixed with pins on the leaves of the potted plant without removal any wing scales deposited by the female *C. perspectalis* during oviposition. All plants were prepared in this way and placed into the cages just prior the experiment was started. Control egg masses were held under same conditions to evaluate host egg quality, i.e. vitality of offered egg masses. For this purpose, twenty *C. perspectalis* egg masses were incubated without parasitoids in glass tubes under the same experimental laboratory conditions.

A few days before the experiment, egg cards with parasitized *Sitotroga cerealella* eggs (about 300 eggs) were inserted singly into glass tubes (10 cm x 2.5 cm diameter). As soon as *Trichogramma* started to hatch, each of these glass tubes were positioned in the middle of the host plant of a cage and opened. A stripe of parafilm with drops of honey was added to the cage for feeding the parasitoids. To approximate the number of wasps released into the cages and their sex ratio, *Trichogramma* from parasitized egg cards of the same rearing batch were held in glass tubes and counted. After four days, the offered *C. perspectalis* egg masses were collected from the box plants and further incubated separately in small glass tubes (25 °C, 60% RH, 16 h light: 8 h dark). After successfully emergence of the *Trichogramma* progeny, two parameters were assessed for evaluating the capacity of the parasitoids for success in host location: (1) percentage of parasitized eggs and (2) percentage of located egg masses per cage (with ≥ 1 parasitized eggs). The parasitism rate was calculated by relating the total number of parasitized eggs to the total number of offered eggs per cage. Additionally, completely parasitized egg masses, non-viable eggs as well as eggs from which *C. perspectalis* had emerged were counted.

Data analysis

Statistical analysis was performed by using the statistical program SAS® Analytics software (Version 9.3). Female-based count data obtained in the host acceptance tests were analyzed by general linear models (GENMOD procedure) with species as fixed and bioassay as the random effect, assuming a negative binomial error distribution with log link in the data set. LSD (Least Significant Difference) tests with Tukey-Kramer adjustment were used for multiple comparisons of differences between species. Cohort-based count data or proportions were analyzed by GLM using poisson, negative binomial or binomial error distribution with species as a fixed effect. Data from the host location test were analyzed by t-test for differences between the two *Trichogramma* species after passing the Brown-Forsythe test for homogeneity of variances and Shapiro-Wilk test for normal error distribution.

Results

Host acceptance

Female-based parameters

Observed number of female egg encounters in a four hour time period differed significantly among species (n = 120, GLM with negative binomial error distribution; χ^2 = 99.10; df = 7; P < 0.0001). On average, females of *T. pintoi* had the most egg encounters (4.32 ± 0.24 encounters/female, P < 0.0001), whereas *T. cacoeciae* contacted the eggs less frequently than all other species (0.89 ± 0.14 encounters/female, P < 0.001). The number of parasitized eggs/female differed significantly among species (n = 120, GLM with negative binomial error distribution; χ^2 = 70.40; df = 7; P < 0.0001). Females of *T. dendrolimi* parasitized significantly more eggs during the exposure time of 72 h than all other species (3.63 ± 0.33 eggs/female, P < 0.01) with a maximum of 21 eggs in one replication. Egg parasitism was lower for the other species, on average less than two eggs/female, although exceptions with higher numbers of parasitized eggs per female occurred in all species (Table 8).

Cohort (bioassay)-based parameters

Mean percentage of active females per bioassay differed significantly among species (GLM with binomial error distribution, corrected for overdispersion; F = 4.33; df = 7, 24; P = 0.0032). More than 85% of the tested *T. pintoi* females and more than 50% of *T. nerudai*, *T. dendrolimi*, *T. brassicae*, *T. bourarachae* and *T. evanescens* females visited egg masses of *C. perspectalis* at least once during the 4 h observation period. In contrast, females of *T. cordubensis* and *T. cacoeciae* were less active than females of the other species (Table 8). Percentage egg parasitism varied considerably in the

bioassays, but was also affected by species level (GLM with binomial error distribution, corrected for overdispersion; F = 2.41; df = 7, 24; P = 0.0509). Here, *T. dendrolimi* achieved the highest parasitism rate (44.2 ± 9.73%, P < 0.0001). In contrast, only 4% (*T. cordubensis*) to 20% (*T. brassicae*) parasitized eggs were determined for the other species (Table 8). The host cluster acceptance rate (HCAR) per bioassay was not significantly different among species (GLM with binomial error distribution, corrected for overdispersion; F = 1.96; df = 7, 24; P = 0.1031). Nevertheless, *T. dendrolimi* attacked most of the offered egg masses (mean HCAR > 70%), followed by *T. brassicae* (mean HCAR > 40%). The lowest HCAR was determined for *T. cordubensis* that attacked about 22% of the offered egg masses (Table 8). *T. dendrolimi* obtained the maximum number of completely parasitized egg masses per bioassay in one test (14 out of 30 offered egg masses) as well as the highest average value of seven completely parasitized egg masses/ bioassay (Table 8). All other species displayed much lower values, ranging between zero (*T. cordubensis*) and two (*T. bourarachae*) completely parasitized egg masses (GLM with negative binomial error distribution, corrected for overdispersion; F = 3.61, df = 7, 24; P = 0.0085). The average rate of non-viable eggs of *C. perspectalis* (28%) was comparable to that observed in the control treatment (36%).

Ranking of species

All species were ranked for their values presented in Table 8 and the results of this ranking are displayed in Table 9. Observed egg encounters were most numerous for *T. pintoi* which showed also the highest percentage of active females, but this species performed less well regarding the other criteria. On the other hand, *T. dendrolimi* produced the highest number of parasitized eggs per female, the highest percentage of parasitized eggs per bioassay, the highest mean of HCAR as well as the highest number of completely parasitized egg masses. Thereafter, *T. dendrolimi* achieved the maximum average rank (2.2) and the first ranking order, followed by *T. brassicae* (2.7). Overall, *T. cordubensis* performed lowest regarding most criteria (average rank 7.7), being less successful in parasitizing *C. perspectalis* eggs (Table 9).

Table 8 Host acceptance based on two female-based and four cohort-based evaluation parameters (mean ± SE): number of encounters per female ($P < 0.05$), number of parasitized eggs per female ($P < 0.05$) and percentage of active females ($P < 0.05$), percentage of parasitized eggs ($P < 0.005$), HCAR[2] (not significant (n.s.)) as well as the number of completely parasitized egg masses per experiment. Different letters indicate significance between species.

Species[1]	female-based parameter				cohort-based parameter							
	[Σ] of encounters per female		[Σ] of parasitized eggs per female		[%] of active females		[%] of parasitized eggs		[%] HCAR[2]		[Σ] of completely parasitized egg masses	
DEN	2.00 ± 0.19	cd	3.63 ± 0.33	a	67.5 ± 5.51	abc	44.2 ± 9.73	a	70.0 ± 13.02	n.s	7.00 ± 2.49	a
BRA	3.12 ± 0.28	abc	1.50 ± 0.24	b	63.4 ± 11.31	abc	20.4 ± 9.97	b	41.7 ± 7.16	n.s	1.50 ± 0.65	ab
PIN	4.32 ± 0.24	a	1.10 ± 0.20	bc	88.3 ± 5.18	a	13.4 ± 9.92	bc	35.0 ± 14.50	n.s	0.25 ± 0.25	b
NER	3.35 ± 0.25	ab	0.84 ± 0.16	bcd	75.0 ± 5.01	ab	11.1 ± 3.81	bc	28.3 ± 7.39	n.s	1.25 ± 0.63	ab
BOU	2.38 ± 0.24	bc	1.04 ± 0.21	bcd	59.1 ± 6.99	abc	14.0 ± 5.37	bc	27.5 ± 8.86	n.s	2.25 ± 1.03	ab
EVA	2.19 ± 0.24	bc	1.17 ± 0.18	bc	56.7 ± 7.58	abc	15.9 ± 7.51	bc	38.4 ± 3.80	n.s	1.00 ± 0.58	b
CAC	0.89 ± 0.14	e	1.22 ± 0.23	bc	38.3 ± 11.98	c	14.5 ± 14.39	bc	33.3 ± 7.85	n.s	0.50 ± 0.50	b
COR	1.97 ± 0.24	cd	0.49 ± 0.12	d	46.7 ± 4.08	bc	4.2 ± 1.09	c	21.7 ± 5.18	n.s	0.00 ± 0.00	c

[1] full name of species and associated labels see Table 7.

[2] HCAR = Host Cluster Acceptance Rate

Table 9 Host acceptance ranking based on two female-based and four cohort-based evaluation parameters (Table 8). Values were ranked from highest to lowest value (1-8) of determined parameters. According to the average rank for every tested species a ranking order was assessed to evaluate the two most effective species against *C. perspectalis* and for further testing.

Species[1]	female-based parameter		bioassay-based parameter				ranking	
	[Σ] of encounters per female	[Σ] of parasitized eggs per female	[%] of active females	[%] of parasitized eggs	[%] HCAR[2]	[Σ] of completely parasitized egg masses	average rank	order
DEN	6	1	3	1	1	1	2.2	1
BRA	3	2	4	2	2	3	2.7	2
PIN	1	5	1	6	4	6	3.8	3
EVA	5	4	6	3	3	5	4.3	4
NER	2	7	2	7	6	4	4.7	5
BOU	4	6	5	5	7	2	4.8	6
CAC	8	3	8	4	5	7	5.8	7
COR	7	8	7	8	8	8	7.7	8

[1] full name of species and associated labels see Table 7.

[2] HCAR = Host Cluster Acceptance Rate.

Host suitability

The emergence rate per parasitized host egg was higher than one for all *Trichogramma* species, thus females tended to lay more than one egg into the eggs of *C. perspectalis*. The species *T. pintoi*, *T. nerudai*, *T. cordubensis*, *T. bourarachae* and *T. cacoeciae* laid 1-2 eggs/host egg on average, whereas *T. evanescens*, *T. brassicae* and *T. dendrolimi* laid 2-3 eggs into one *C. perspectalis* egg. The *Trichogramma* F1 offspring consisted mostly of females so the sex ratio (male: female) was female-biased, except for *T. cordubensis* (male: female = 1.14: 1). *T. dendrolimi* (male: female = 0.35: 1) and *T. bourarachae* (male: female = 0.34: 1) produced even more than 70% females in the eggs of this host. *T. cacoeciae* is an obligate thelytokous species producing 100% females independent of host quality (Figure 12).

Host location

The mean percentage of egg masses located by parasitoids (with ≥ 1 parasitized eggs) on *Buxus* plants was 40% for *T. dendrolimi*, almost twice as high as for *T. brassicae* with 23% (Figure 13). However, this difference was not significant ($t = 1.872$; $df = 15$; $P = 0.081$). The maximum value was observed in one replicate/experiment with six egg masses located out of ten offered to both species. Total egg parasitism was significantly different between the two species ($t = 2.677$; $df = 15$; $P = 0.017$). On average, *T. dendrolimi* parasitized 13% of the offered eggs with a maximum of 30%

parasitism in one cage. *T. brassicae* parasitized even less than 10% of the offered eggs with a maximum at 13%. On average, 56% *C. perspectalis* larvae hatched and about 35% of the host eggs were non-viable after finishing the experiments. Control eggs resulted on average in a successful emergence of 85% *C. perspectalis* larvae, whereas about 15% of the eggs were non-viable (Figure 13).

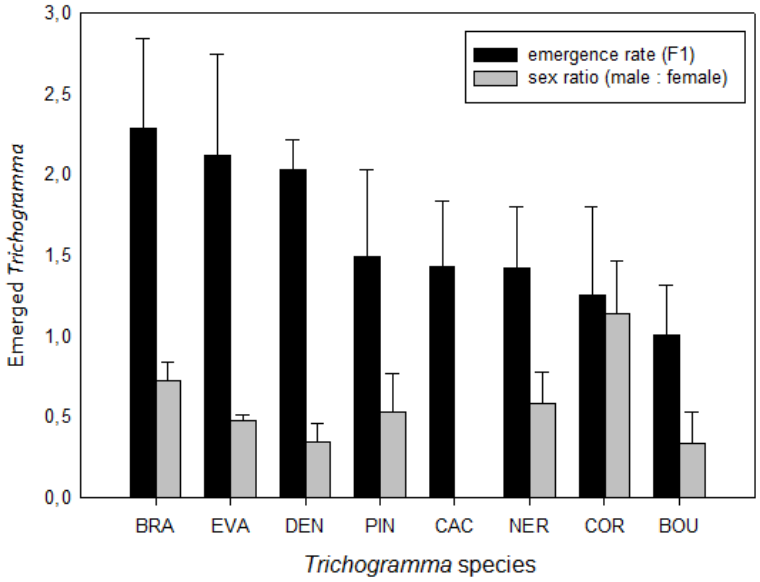


Figure 12 Progeny production (emergence rate and sex ratio (male: female)) to determine the host suitability of the eight tested *Trichogramma* species emerging from parasitized *C. perspectalis* eggs during the host acceptance test. Full name of species and associated labels see Table 7.

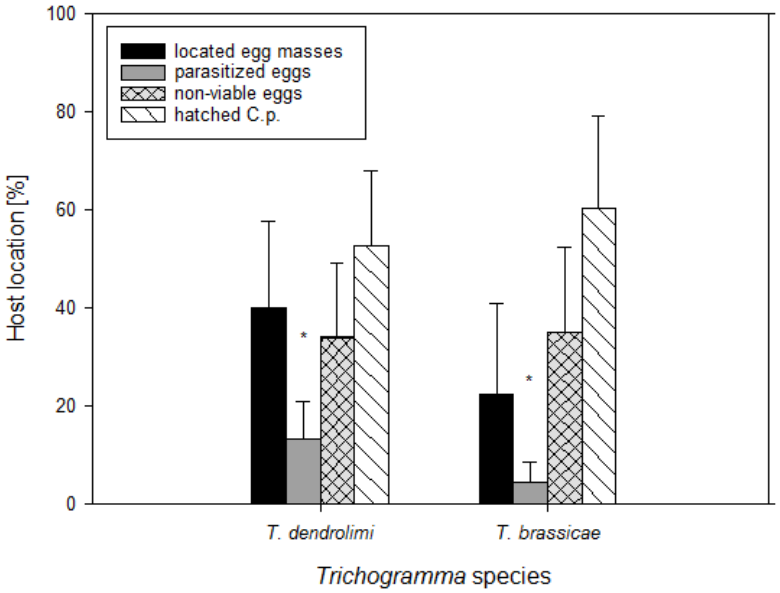


Figure 13 Success of *T. dendrolimi* and *T. brassicae* in locating and parasitizing egg masses of *C. perspectalis* distributed on *Buxus* plants in cage experiments. Percentage of located egg masses (with at least one parasitized egg), percentage of parasitized eggs, percentage of non-viable eggs and the hatching rate of *C. perspectalis* were compared. The asterisks denote significant differences between the two species ($P < 0.05$).

On average 296 ± 62 (n = 7 counted egg cards) *T. dendrolimi* wasps had been released per cage with about 117 ± 26 released females, resulting in an average sex ratio (males: females) of 1.6: 1. On average 290 ± 94 (n = 7 counted egg cards) *T. brassicae* wasps had been released per cage with about 128 ± 46 released females, resulting in an average sex ratio (males: females) of 1.1: 1. About 140 ± 26 host eggs (n = 18 cages) were offered per cage. Accordingly, the parasitoid: pest ratio tended to be close to 1 : 1, the ratio of females: host egg mass was 14 : 1.

Discussion and conclusion

Initial studies on the natural regulation of *C. perspectalis* in Europe were not very promising. In China and Japan, some common natural enemies were described, such as Diptera (Tachinidae) and Hymenoptera (Braconidae, Chalcididae, Encyrtidae, Ichneumonidae) and predators such as Thysanoptera (Aeolothripidae) and spiders (Araneae). But their impact on *C. perspectalis* populations remains low and mostly unknown. A more specific and well adapted egg-larval parasitoid, *Chelonus tabonus* Sonan (Braconidae), was found in China but its host specificity and its potential introduction into Europe as classical biological control agent has to be further investigated (Wan et al. 2014). First experiences using the commercially available larval parasitoids *Bracon brevicornis* Wesmael and *Bracon hebetor* Say (Braconidae) were done in the laboratory as well as in semi field trials, however these parasitoids could not complete their development in this new host (Zimmermann & Wührer 2010). In Switzerland, the only native natural enemy found so far was *Pseudoperichaeta nigrolineata* Walker (Tachinidae) but the low parasitism rates indicate that they lack the ability to regulate *C. perspectalis* populations in Europe (CABI 2013). Also single Box tree pyralid larvae parasitized by a tachinid fly or predated by the polyphagous bug *Orius majusculus* Reuter as well as by spiders were observed in the field in Germany, but their regulatory impact is probably moderate (unpublished data). No *Trichogramma* wasps were found as natural enemies of *C. perspectalis* in China (Wan et al. 2014) so far and very little research on the use of *Trichogramma* spp. for the biological control of *C. perspectalis* has been done. First trials with *T. brassicae* demonstrated the ability of *Trichogramma* wasps to parasitize eggs of *C. perspectalis* in the laboratory (Zimmermann et al. 2009) and releases of a commercial *Trichogramma* product in a nursery were perceived by the farmer to cause lower pest pressure, although parasitism or pest control was not quantified (Zimmermann et al. 2009; Albert & Lehneis 2010).

The present study suggests that females of various *Trichogramma* species may accept *C. perspectalis* eggs as a potential host. More than 50% of females of most species encountered the eggs and displayed antennal drumming and drilling into eggs for oviposition during the 4 h observation

period. There is only one layer present in an egg mass of *C. perspectalis* and the round-shaped eggs are flat with slightly overlapping edges. Thus, *Trichogramma* wasps do not have to penetrate a second or third layer for oviposition and have a direct access to every offered egg of the mass. They were able to place up to three eggs into one *C. perspectalis* egg. Successful progeny production also indicated that *C. perspectalis* could be a suitable host, because emergence rates from parasitized eggs were high (up to 2-3 parasitoid eggs/host egg) and sex ratio was female-biased. It is well known that host quality influences clutch size (i. e. number of eggs/host) and sex ratio of arrhenotokous parasitic Hymenoptera, thus allowing the optimal use of the host resource by the parasitizing female for progeny production (King 1987; Godfray 1994). Our findings suggest that ovipositing females evaluate the host quality of *C. perspectalis* positively by laying several, mainly fertilized female eggs into this host resource. IOBC standards on quality control for mass-reared natural enemies request ≥ 50% female offspring in mass production units of *Trichogramma* (Hassan & Zhang 2001) for sufficient parasitoid quality. This criterion was met for nearly all tested *Trichogramma* species in our trials (except *T. cordubensis* with a sex ratio of 1.14).

The host acceptance ranking indicated that *T. dendrolimi* was the most successful tested *Trichogramma* species within this investigation. This species was ranked first for four of the six evaluation criteria parameters, because it achieved the highest number of parasitized eggs per female (3.63), the maximum percentage of parasitized eggs (44%), the highest host cluster acceptance rate (70% attacked egg masses) and the highest number of completely parasitized egg masses (7 out of 30 per bioassay). Furthermore, it produced a highly female-biased progeny with 2 to 3 emerged wasps per host indicating host egg suitability. Nevertheless, all these results do not comply with IOBC standards for parasitoid quality as requested for mass produced *Trichogramma* (Hassan & Zhang 2001), e.g. sufficient rates of host acceptance must be on average ten successfully parasitized host eggs in 4 h and the host cluster acceptance rate should be at least 80%. In our study, even T. *dendrolimi* parasitized on average less than five eggs and at maximum 21 eggs within 72 h. The host cluster acceptance rate for this species was only 70% and for the other species even lower. *Trichogramma* females will usually stay on or near located 'preferred' host eggs for a long period of time until all of them are parasitized (Hassan 1993). However, in our study, the number of completely parasitized egg masses was very low for all species (≤ 2/30 per experiment). Surprisingly, the species with the highest percentage of active females and most egg encounters per female (*T. pintoi* and *T. nerudai*) were not the species reaching the highest parasitism levels. Considering the high egg encounter rate in general but the low parasitism capacity thereafter in all species, we

suggest that there must be factors probably impeding successful parasitism at the beginning of the oviposition or during initial parasitoid development.

Factors like parasitoid and/or host age may influence the parasitism rate and the progeny production of parasitoids (Pak & Dejong 1987; Godfray 1994). In the case of *Trichogramma*, most studies report decreasing host suitability or reduced host preference of parasitoids with increasing age of the host eggs. For instance, Farag et al. (2013) showed this effect for *T. cacoeciae*, *T. evanescens* and *T. bourarachae* on eggs of *Ephestia cautella* Walker with parasitism rates of more than 70% on 0 h to 24 h old eggs and of less than 30% on eggs older than 24 h. Also Ko et al. (2014) reported higher parasitism rates and progeny production on eggs of the Crambid *Chilo suppressalis* Walker which were 0 h to 24 h old in comparison to eggs older than 24 h. On the other hand, Herz and Hassan (2006) conducted host acceptance tests with eggs of *Palpita unionalis* Hübner which were between 24 h and 48 h old, because preliminary tests had shown that parasitism of younger eggs was less successful. In contrast, Liu et al. (1998) showed that parasitism capacity of *T. dendrolimi* on eggs of Asian Corn Borer *Ostrinia furnacalis* Guenée dropped significantly from 45% (egg age between 0 h to 6 h) to 18% and less when eggs were older than 18 h. Thus, even a short time period of a few hours may alter the host suitability (Liu et al. 1998). We did not explicitly tested susceptibility of *C. perspectalis* eggs of different ages to parasitism by the different *Trichogramma* species previously.

Another possibility for low parasitism rates despite high egg attractiveness to *Trichogramma* females could be host feeding. *Trichogramma* species display host feeding, especially on the first eggs encountered after oviposition which can influence size and fitness of emerging progeny. Depending on the intensity of host feeding, hosts can also dry out (Ferracini et al. 2006). In the present study, the amount of non-viable host eggs was in the range of control eggs in the host acceptance test (both around 30% of eggs), but it was higher in the host location test (control eggs: 15%, *Trichogramma* exposed eggs: 35%). This observation suggests that some of the hosts had experienced host feeding and/or parasitization, rendering eggs non-viable. Pak et al. (1990) found differences in susceptibility to desiccation after parasitisation in eggs of *Mamestra brassicae* Linnaeus and *Pieris brassicae* Linnaeus. He concluded that the structure and permeability of the specific host chorion influenced the water-loss resistance of the egg and consequently the success of the parasitoid's development.

Trichogramma is an idiobiont endoparasitoid and the venom of the ovipositing female usually kills the host embryo. It is surprising that no *Trichogramma* species were found attacking *C. perspectalis* in its native range, although the Asian fauna of Trichogrammatidae is species rich and well known

(Polaszek 2010). Important factors for the acceptance of host eggs by parasitoids are physical factors like shape, size, movement and sound in conjunction with chemical compounds on and inside the eggs like odors for chemosensory stimulation, toxins, cytolytic enzymes and nutrients (Vinson 1976; Vinson & Iwantsch 1980). *Buxus* plants contain highly toxic secondary plant metabolites (alkaloids). Leuthardt et al. (2013) investigated the uptake to *C. perspectalis* larvae and determined that they stored large amounts of dibasic alkaloids in their body. Newly emerged adult moths contained no traces of alkaloids, but there are no reports on whether eggs contain these chemicals or not. It is also possible, that unsuccessful parasitism was due to biochemical or physiological interactions between host and the developing parasitoid in terms of immune-based defense like encapsulation or melanization. Reed et al. (2007) described this for a Coleoptera-Encyrtidae system where newly hatched larva of the egg parasitoid *Avetianella longoi* Siscaro were successfully inactivated by the host *Phoracantha recurva* Newman during the egg stage. Kanost et al. (2004) found binding proteins in *Manduca sexta* Linnaeus (Lepidoptera) eggs which trigger diverse responses such as phagocytosis, nodule formation, encapsulation, melanization and synthesis of anti-microbial proteins for defense. In addition, Abdel-latief and Hilker (2008) pointed out that *Manduca sexta* Linnaeus could defend itself from attack from *Trichogramma* by enhancing transcription enzymes in the host egg as an immune response. Therefore, chemical or immune-based defense mechanisms might also occur in eggs of *C. perspectalis*, which impede successful development of the parasitoid.

Results of the host location test indicated that *Trichogramma* females were able to walk on the *Buxus* plant leaves and to locate egg masses, mainly deposited on the lower leaf side. Thus, successful host location on the plant, one prerequisite for the utility of a biocontrol agent in plant protection, is possible. The two species chosen for this test, *T. dendrolimi* and *T. brassicae,* are native in Germany and other parts in Europe and they are commercially available. These facts would be highly advantageous for their use as biocontrol agents for regulation of the Box tree pyralid in the newly invaded area. However, the low parasitism rates and the low number of completely parasitized egg masses in spite of the high numbers of released *Trichogramma* wasps (14 released females per offered host egg mass) indicate insufficient control efficacy and prevention of larval hatching and subsequent damage, at least in this experiment.

In general, this study has increased our knowledge of the potential for controlling *C. perspectalis* using *Trichogramma*. There was a considerable acceptance of *C. perspectalis* eggs as hosts for various *Trichogramma* species. Female parasitoids successfully oviposited on these eggs and produced viable offspring. Host location on *Buxus* plants was possible, but resulting host parasitism

was unexpectedly low. Unfortunately, the eight *Trichogramma* species tested in this study did not meet standards set by IOBC for efficient parasitism. It is certainly valuable to screen other strains or species of this genus recalling the case of *O. nubilalis* Hübner that had required several decades of species selection until the biocontrol program against European Corn borer was successful. Nevertheless, it is even more important to analyze the host-parasitoid interaction within the host egg in order to prove the assumption of potent chemical or immune-based defense of *C. perspectalis* against egg parasitoids like *Trichogramma*.

Chapter III.

Prey acceptance and location of *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) eggs by *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae)

Abstract

Experiments on the prey acceptance and prey location of *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) should clarify to which extent the commercially available beneficials *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae) do prey on *C. perspectalis* eggs. Due to their polyphagy they were taken into consideration to be potential predators of *C. perspectalis.* Their release might be a suitable tool for supporting its biological control. The performance of *C. carnea* was significantly better in comparison to *O. majusculus*. *C. carnea* preyed on average 35.8 ± 12 of 40.1 ± 8.9 (89%) offered eggs during the prey acceptance bioassay (72 h) in experimental vials and 92.6 ± 23 of 189.4 ± 15.8 (49%) offered eggs during the prey location on the *Buxus* plant. In contrast, *O. majusculus* preyed 8.9 ± 5.9 of 21.9 ± 7.1 (41%) offered eggs during 72 h of direct egg exposure and 13.8 ± 8.4 of 102.7 ± 16.9 (13%) offered eggs were located and preyed on the plant. Number of located egg masses on the plant did not significantly differ between the species. Thus, both tested species accepted *C. perspectalis* as a host and preyed it in different extent. The augmentation of native natural enemies as a tool in biological control might be considered in the case of *C. perspectalis*. *C. carnea* larvae are the more voracious predators, but their natural occurrence on *Buxus* plants is rather low. Therefore, consideration may be given for an inundative release of larvae of these native and commercially available beneficials. Since the natural occurrence of *O. majusculus* on *Buxus* plants and the lesser voraciousness, their protection on the *Buxus* plant might be more profitable than their release. Thus, these and other natural occurring predators can be preserved by avoiding the use of chemical plant protection products and might benefit the biological regulation of *C. perspectalis* field populations.

Keywords: beneficials, *Buxus*, biological control, prey acceptance, prey location

Introduction

This study was addressed to find an environmentally and user friendly as well as effective method to control *C. perspectalis*, which does not require permitting or testing for introduction of exotic biological control agents or their application. Many predatory bugs and spiders are present in *Buxus* plants (personal observation). Egg predators prevent hatching of larvae, thus reducing the risk for defoliation. Additionally, they have probably not to deal with toxins like dibasic alkaloids, which are accumulated in the body of *C. perspectalis* larvae (Leuthardt et al. 2013). Experiments on the prey acceptance and location of *C. perspectalis* eggs should clarify to which extent the native as well as commercially available beneficials *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae) do prey on *C. perspectalis* eggs.

While *C. carnea* adults feed on nectar, pollen and aphid honeydew, larvae are active predators that prefer feeding on aphids but are also feeding on spider mites, mealy bugs and lepidopteran eggs. They have been used in the biological control of insect pests on crops, especially on vegetables and ornamentals in the greenhouse (Bay et al. 1993; Jehle et al. 2014). *O. majusculus* is a generalist that attacks a variety of pests. Their prey spectrum includes psyllids, thrips, aphids, spider mites, as well as lepidopteran eggs (Lattin 1999; Jehle et al. 2014). Both predators occur naturally in a wide range of habitats. As generalists and polyphagous species that prey on lepidopteran eggs, they were taken into consideration to be potential predators of *C. perspectalis* eggs and their release might be a suitable tool for supporting its biological control. Furthermore, they are already commercially produced and available on the market, which would allow an easy and quick application in practice.

Material and methods

C. perspectalis eggs

C. perspectalis have been reared under laboratory conditions since 2010. Larvae were fed on watered box tree twigs (*Buxus sempervirens* var. arborescens Linnaeus) in transparent Perspex cylinders (height 40 cm × diameter 19.5 cm) with textile covers. Adult moths were kept in rearing cages (length 40 cm × width 36 cm × height 50 cm) with diluted sugar (5%) for feeding. Short box tree twigs (15–20 cm) were regularly introduced into the cages for egg deposition. Single leaves, each with one *C. perspectalis* egg mass (≤ 24 h old) were collected from the box tree twigs for the experiments. Females of *C. perspectalis* deposited the egg masses during the preceding night. A standardisation of the number of eggs per leaf was impossible, since removing eggs from a

clustered egg mass or any other manipulation of the egg mass might cause alteration in the signal structures of the eggs which serve as attractants for predators and are important for the host finding, like the emittance of kairomones and other chemical cues.

Chrysoperla carnea larvae

Larvae of Common green lacewing *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) were reared under laboratory conditions. Adults were kept in rearing boxes (length 25 cm × width 25 cm × height 15 cm) covered with mull for egg deposition and fed on an artificial diet, consisting of wheat germs, beer yeast, fructose, milk and honey. Larvae (Figure 1) were reared in plastic boxes (length 40 cm × width 36 cm × height 15 cm) covered with wire gauze and fed on eggs of the Angoumois grain moth *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) obtained from BIOCARE GmbH (Dassel-Markoldendorf, Germany). Larvae were isolated and starved for 24 h before conducting the experiments.

Orius majusculus adults

O. majusculus Reuter (Heteroptera: Anthocoridae) nymphs were provided by the company Katz Biotech AG (Baruth, Germany) and further reared in aerated storage boxes (length 19.5 x width 19.5 x height 9.5 cm). They were fed on eggs of the Angoumois grain moth *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) obtained from BIOCARE GmbH (Dassel-Markoldendorf, Germany). Runner beans were regularly introduced for egg deposition. Adults (Figure 14) were isolated and starved for 24 h before conducting the experiments.

Figure 14 *O. majusculus* adult (left), *C. carnea* larva on *C. perspectalis* egg mass (middle), *H. axyridis* larva and adult feeding on *C. perspectalis* larvae (right).

Buxus plants

Plant material (*Buxus sempervirens* var. arborescens Linnaeus) was obtained from a box tree nursery (Baumschule Atrops, Rheudt, Germany). For rearing cut twigs (30 cm), for the direct exposition single leaves and for prey location experiments, potted *Buxus* plants (40–50 cm) were utilized.

Prey acceptance

Prey acceptance of *C. carnea* larvae and *O. majusculus* adults was explored to evaluate if *C. perspectalis* eggs are potential prey to the investigated predators. Therefore, bioassays with direct exposition of *C. perspectalis* egg masses to predator individuals were carried out in the laboratory. One bioassay included 10 replicates (= vials) and was performed three times. One replicate consisted of one individually caged predator in a small experimental vial (height 3 cm x diameter 3.5 cm) containing three *Buxus* leaves, each with a host egg mass (≤ 24 h old), as well as a wet cotton pallet for providing a water source. Vials were incubated at 25°C, 16/8 h L/D, 60% RH. After 72 h of exposure offered egg masses were observed in detail with a stereo microscope. Preyed eggs were distinguished by bite marks with dark discolorations. Hatched eggs can easily be recognized as empty. The chorions remain transparent and retain their primary structure, except of the clearly visible holes, where the larvae hatched out. The rate of predation was calculated by relating the number of preyed eggs per vial to the number of offered eggs per vial. In addition, the proportion of egg masses with at least one preyed egg was determined because predated eggs were not uniformly distributed on the offered egg masses.

Prey location

Prey location of *C. carnea* and *O. majusculus* was explored to analyse the ability of both predators to locate the *C. perspectalis* eggs on the plant and feed on them. Therefore, cage experiments were conducted with potted *Buxus* plants (*B. sempervirens* var. arborescens) at 25°C, 16/8 h L/D, 60% RH in the laboratory. Each experimental run included six replicates (= cages) per predator species and was performed three times. For each cage (length 65 cm × width 50 cm × height 100 cm), 10 leaves with egg masses (≤ 24 h old) were distributed on one potted *Buxus* plant. The leaves with egg masses were carefully fixed with pins on the leaves of the potted plant. All plants were prepared in this way and placed into the cages just prior to initiating the experiment. Five predators were applied per plant and few cotton pallets were added for providing a water source. After 72 h of exposure egg masses were collected and observed in detail with a stereo microscope. Preyed eggs were distinguished by bite marks with dark discolorations. Hatched eggs can easily be recognized as empty. The chorions remain transparent and retain their primary structure, except of the clearly

visible holes, where the larvae hatched out. The rate of predation was calculated by relating the number of preyed eggs per cage to the number of offered eggs per cage. In addition, the proportion of egg masses with at least one preyed egg was determined because predated eggs were not uniformly distributed on the offered egg masses.

Statistical analyses

For statistical analyses the scientific data analysis software SigmaPlot was used (Version 13.0, from Systat Software, Inc., San Jose California USA). Normality and equal variance of the data were tested by Shapiro-Wilk and Brown-Forsythe tests. Number of preyed eggs and egg masses were compared between the two predator species by conducting t-tests (α = 0.05) based on three experimental runs of the prey acceptance and prey location trials.

Results

Prey acceptance bioassay

C. carnea preyed on average 35.8 ± 12 eggs of 40.1 ± 8.9 offered eggs per vial, i.e. individual predator within 72 h of direct exposure. Thus, mean egg predation was almost 90%. This was a significantly better performance than the performed egg acceptance by *O. majusculus*, which preyed the lesser amount of 8.9 ± 5.9 eggs out of 21.9 ± 7.1 offered eggs per experimental vial (t = 5.12, df = 4, p = 0.007). Based on the number of preyed eggs, the predation rate of *O. majusculus* per 72 h and individual predator reached only a quarter of the predation rate demonstrated by *C. carnea*. Preyed egg masses per vial with at least one preyed egg were 2.6 ± 0.6 by *C. carnea* and 1.9 ± 0.9 by *O. majusculus*. In this case, predation did not significantly differ between the two predators (t = 2.5, df = 4, p = 0.06) (Table 10).

Prey location on the plant

C. carnea larvae preyed on average 92.6 ± 23 eggs of 189.4 ± 15.8 offered eggs per cage within 72 h exposure to five applied predators on the plant, i.e. 19 eggs/ individual predator. This performance was significantly better than the performed egg location by *O. majusculus*, which preyed 13.8 ± 8.4 eggs from 102.7 ± 16.9 offered eggs per cage, i.e. 3 eggs/ individual predator (t = 5.28, df = 4, p = 0.006). Preyed egg masses with at least one preyed egg (of 10 exposed) were 5 ± 1.5 by *C. carnea* and 3.3 ± 1.5 by *O. majusculus*, whereas a maximum of ten egg masses offered per cage were preyed. In this case, predation rate did not significantly differ between the two predators (t = 1.4, df = 4, p = 0.24) (Table 11).

Table 10 Offered and preyed eggs per vial, i.e. individual predator (*C. carnea* and *O. majusculus*) within 72 h of exposure in vials, conducted three times (1-3). Letters show significant differences between the two tested species (t-test, α = 0.05).

	offered eggs per egg mass (n = 30 masses)	**offered eggs per vial** (n = 10 vials)	**preyed eggs per vial** (n = 10 vials)	**preyed eggs [%]**	**preyed egg masses per vial** (max. 3)
O. majusculus (1)	9 ± 5.7	27.0 ± 6.8	6.2 ± 4.9	23.0	1.6 ± 0.7
O. majusculus (2)	8 ± 6.5	23.1 ± 10	13.1 ± 8.9	56.5	2.1 ± 0.9
O. majusculus (3)	5 ± 2.2	15.6 ± 4.7	7.4 ± 4.0	47.4	2.1 ± 1.0
mean	**7.3 ± 4.8**	**21.9 ± 7.1**	**8.9 ± 5.9 a**	**42.3 ± 17.3**	**1.9 ± 0.9 a**
C. carnea (1)	15 ± 7.9	45.4 ± 9.9	43.8 ± 10.6	97.8	2.9 ± 0.3
C. carnea (2)	10 ± 5.2	30.4 ± 10.1	27.2 ± 8.9	90.0	2.8 ± 0.4
C. carnea (3)	14 ± 6.2	44.4 ± 6.8	36.4 ± 16.6	81.8	2.2 ± 1.2
mean	**13.0 ± 6.4**	**40.1 ± 8.9**	**35.8 ± 12 b**	**89.9 ± 8.0**	**2.6 ± 0.6 a**

Table 11 Offered and preyed eggs per cage, i.e. per five predators (*C. carnea* and *O. majusculus*) within 72 h of exposure on the plant and preyed eggs per individual predator, conducted three times (1-3). Letters show significant differences between the two tested species (t-test, α = 0.05).

	offered eggs per egg mass (n = 60 egg masses)	**offered eggs per cage** (n = 6 cages)	**preyed eggs per cage** (n = 6 cages)	**preyed eggs per predator**	**preyed eggs [%]**	**preyed egg masses** (max. 10)
O. majusculus (1)	12 ± 6.3	120 ± 18.4	3 ± 3.3	1 ± 0.6	2.1	1 ± 1.1
O. majusculus (2)	12 ± 7.2	115 ± 18.5	26 ± 14.4	5 ± 2.9	22.9	5 ± 1.3
O. majusculus (3)	7 ± 4.7	73 ± 13.9	13 ± 7.5	3 ± 7.5	17.1	4 ± 2,1
mean	**10.3 ± 6.1**	**102.7 ± 16.9**	**13.8 ± 8.4 a**	**2.8 ± 1.7**	**9.6 ± 10,6**	**3.3 ± 1.5 a**
C. carnea (1)	20 ± 10.4	200 ± 33.7	95 ± 45.7	19 ± 9.1	47.5	5 ± 1.8
C. carnea (2)	22 ± 14.6	197 ± 80.1	115 ± 59	23 ± 11.8	58.4	5 ± 1.5
C. carnea (3)	17 ± 8.6	171 ± 47.9	69 ± 24.2	14 ± 4.8	40.4	5 ± 1.3
mean	**19.7 ± 11.2**	**189.4 ± 15.8**	**92.6 ± 23 b**	**18.5 ± 4.6**	**48.8 ± 9.1**	**5.0 ± 1.5 a**

Discussion

In this study, *C. carnea* and *O. majusculus* preyed successfully on eggs of *C. perspectalis*. Both tested beneficials stayed on the *Buxus* plants during the cage experiments and were able to locate *C. perspectalis* eggs on it. *C. carnea* larvae were the more voracious predators and their performance was significantly better than the capacity of *O. majusculus* in experimental studies on prey acceptance and prey location on the *Buxus* plant.

The performance of both species might be affected by the quality of host eggs or factors like prey density. Host egg quality was estimated to be adequate because both species used the offered eggs as food source. Furthermore, *O. majusculus* utilized the offered *C. perspectalis* eggs for their own egg deposition, to supply the offered food source additionally to their offspring. In addition, *C. perspectalis* larvae hatched out of unpreyed eggs, indicating vitality. The amount of prey of a target species is depending on the densities of the prey and predator populations as well as their characteristics e.g. stimulus detected by predator, food preferences and efficiency of attack. In addition, density and quality of alternate foods available for the predator are important facts on predation (Holling 1959). At low densities, number of prey is limited by the search time, thus a predator spends most of his time searching for potential prey. The situation is different at high prey density because a predator has to spend only little time on searching the target. At high prey density the number of prey is limited by the handling time or the degree of predator saturation (Nentwig et al. 2007). In our study, during the direct exposure of eggs in small experimental vials, no time for searching was necessary, prey on *C. perspectalis* was rather limited to saturation or the basic willingness to accept the potential offered prey. Prey density on the *Buxus* plants was rather low and beneficials had to spend their main time for searching the target. If this prey density would be also the case in field and the searching activity of the predator is effective, the possibility for the beneficial would be given to keep the field population low and to delay or prevent their rise. But if *C. perspectalis* populations are occurring at high density levels in the field, the impact of egg predators might not be sufficient to adequately reduce the population and the extent of damage to the plant.

In the case of *Buxus* plants in the field, the probability that *C. carnea* will naturally occur on it is rather low. The attraction for *C. carnea* adults to settle and reproduce on the plant is probably not great enough, since *C. carnea* adults feed on honeydew, nectar and pollen and are mostly attracted by aphid populations, which are not found on *Buxus*. In contrast, *O. majusculus* nymphs and adults are active predators. The occurrence of various species of the suborder Heteroptera (including the family Anthocoridae) on *Buxus* plants was already observed in the field as well as the predation of *C. perspectalis* larvae by polyphagous predators like spiders. Additionally *Harmonia* spp. (Coleoptera:

Coccinellidae) larvae and adults were observed occurring naturally in large amounts on *Buxus* plants. *H. axyridis* Pallas (Coleoptera: Coccinellidae) (Figure 14) preyed *C. perspectalis* eggs and larvae at high rates in a test series in our laboratory (Poloczek 2012).These and other predators could have been lured by the presence of potential prey like psyllids and other leaf suckers occurring in the *Buxus* plant. Therefore, it is probable that *O. majusculus* and other polyphagous predators prey on *C. perspectalis* populations in the field.

This study makes clear that augmentation and conservation of natural enemies as tools in biological control might be considered in the case of *C. perspectalis*. *C. carnea* larvae are the more voracious predators, but their natural occurrence on *Buxus* plants is rather low. Therefore, consideration may be given for an augmentation by an inundative release of larvae of these native and commercially available beneficials. Because of the natural occurrence of *O. majusculus* on *Buxus* plants and their lesser voraciousness, while they are more expensive in comparison to *C. carnea*, their protection and conservation on the *Buxus* plant might be more profitable than their release. Thus, these and other natural occurring predators might be preserved by avoiding the use of chemical plant protection products like pyrethroids and neonicotinoids, because they can be very disruptive to beneficials and ecosystems (Dağlı & Bahşi 2009; EASAC 2015). The application of biological control agents might benefit the natural regulation of *C. perspectalis* field populations by natural enemies and could be further enhanced by the release of *C. carnea* larvae as a biological control measure.

Chapter IV. GÖTTIG S. & HERZ A. *Journal of Plant Diseases and Protection* (in preparation)

Susceptibility of the Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) to potential biological control agents Neem (NeemAzal®-T/S) and Entomopathogenic nematodes (Nemastar®) assessed in laboratory bioassays and field trials

Abstract

The Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) is one of the accidentally introduced organisms that became invasive and established in its new environment. Within a few years, it became a serious pest in Germany and many European countries. Thus, efficient pest management is required. Eco-friendly preparations, which should be accessible, affordable, easy usable and suitable for the application in private and public areas, are needed. Therefore, the effects of the plant extract formulation NeemAzal®-T/S (active ingredient Azadirachtin A) and the commercial preparation Nemastar® (Entomopathogenic nematode species *Steinernema carpocapsae*) were investigated on *C. perspectalis* larvae in laboratory bioassays and field trials. In the laboratory, larvae were susceptible to both tested products. When NeemAzal®-T/S treated leaf discs were consumed, a significant effect on mortality and feeding activity was noted after 14 days of exposure. At this time 47%-62% of the larvae had already died and less than 10% of larvae were still feeding. Application of different *S. carpocapsae* suspensions (10-200 EPN/ 100 µl, i.e. per larva) demonstrated a high susceptibility of both tested larval instars (2^{nd}: 10%-75% and 4^{th}: 45%-100% mortality). Infection of further developed larvae was faster. Pupae were much less susceptible to nematodes and only infected in a range of less than 10%. In field trials neither the application of Nemastar® nor the use of NeemAzal®-T/S caused mortality rates comparable to those generated by the treatment with plant protection products based on *Bacillus thuringiensis*. This also applied to the persistence of products on the foliage. In all cases NeemAzal®-T/S caused the lowest mortality and shortest persistence, followed by Nemastar®. In conclusion, there is a higher variability in terms of the effects of the investigated agents Nemastar® and NeemAzal®-T/S compared to the constant effectiveness of *B. thuringiensis*. But their use would be possible if individual feeding damage and proper surveillance will be considered in order to be capable of repeating the application timely. More frequent and timely applied treatments may improve efficacy and a combination with further measures ought to take place.

Keywords: *Buxus*, biological control, feeding activity, larval mortality

Introduction

Organisms are frequently brought into new environments by humans. After importing, the species are capable of reaching different levels of invasion success (introduced, established and pest) as defined by Williamson and Fitter (1996a). Just a small proportion of introduced species have the potential to become established and only a small amount of these established species will become a serious pest (Williamson & Fitter 1996b). The Box tree pyralid *Cydalima perspectalis* (Walker 1859) is one of these accidentally introduced, therefore non-native and invasive organisms that have accomplished an establishment in Germany and Europe. Further, it can be classified as pest, since of causing negative economic effects as well. According to the law, trees belong to the owner of the land on which they grow. Their damage or destruction therefore affects the value of the land of which they form part. Cemeteries and large park facilities with partially several-kilometre-long *Buxus* hedges are strongly affected by a reduction in economic value, depending on feeding damage caused by *C. perspectalis* (Leuthardt 2013). Moreover, *C. perspectalis* continuously spreads (Nacambo et al. 2014) and constantly causes serious damage on ornamental plantings and natural *Buxus* stands in Europe (John & Schumacher 2013).

Buxus is an evergreen perennial shrub (Oberdorfer et al. 2001) of great traditional meaning. It is planted in a large number in private and public areas. It is also very popular on cemeteries for adorning graves and enclosing irrigation systems. Most of the plantings are structurally important subdivision elements. Especially in these public urban areas, the application of chemical insecticides may be often necessary but disliked or forbidden. Therefore, cemeteries are frequently becoming a source of infection of plants growing nearby, due to renewed attack. *C. perspectalis* develops exclusively on plants of the genus *Buxus* and herbivorous competitors are lacking in Europe (Wan et al. 2014). Only a small amount of sucking organisms utilise *Buxus* plants as feeding source. For instance the Boxwood psyllid (*Psylla buxi*), the Boxwood spider mite (*Eurytetranychus buxi*) and diverse scales (van Trier & Hermans 2007), but caused damage is purely aesthetic and not as destructive as by *C. perspectalis* or fungal *Buxus* diseases.

Because of this pest characterisation, efficient pest management is required. Preparations based on *Bacillus thuringiensis* are eco-friendly and specifically effective against lepidopteran larvae. Many strains produce endotoxins as pre-proteins which accumulate in crystalline inclusions in the bacterium. After ingestion, these crystals are dissolved by proteases in the gut of the host insect producing active proteins which interfere with the metabolism of the target insect (Schnepf et al. 1998). Immediate feeding stop occurred by rapid paralysis of mouthparts and gut followed by gut and haemolymph poisoning, which leads to the death of the insect after a few days (Burges 1982;

Schmutterer & Huber 2005). *B. thuringiensis* is highly effective against *C. perspectalis* and registered. However, two main problems arise: If treatment has to be done, public areas have to be closed for visitors until the spray deposit is completely dry (24 h). Furthermore, as the only available biological control measure, the risk of resistance formation exists with frequent application (Schnepf et al. 1998).

Thus, further eco-friendly preparations are needed which should be accessible, affordable, easy to use and suitable for the application in public areas, e.g. cemeteries, monasteries and public parks as well as house gardens. Since the beginning of biological control in the 1980s, numerous publications and reviews have been written and pointed out many advantages of Neem products and entomopathogenic nematodes, regarding insect control (Schmutterer 1990; Kaya & Gaugler 1998; Schmutterer & Huber 2005). These agents are well investigated, commercially available and are used against various pests.

Formulations with Azadirachtin, the most important active ingredient in seeds of the Neem tree (*Azadirachta indica* A. Juss (Meliacea)), were tested and successfully used against various insect pests. Benelli et al. (2017) reviewed the potential of Neem-based products and pointed out, that on the basis of its properties like effective and eco-friendly features, including little non-target effects, multiple mechanisms of action, low cost and easy production, Neem-based products can serve as an advantageous alternative to build newer and safer arthropod control tools. It serves as insect growth regulator and feeding deterrent to many insects (Isman 2006) and there are anti-ovipositional, fecundity- and fitness-reducing properties as well (Schmutterer 1990).

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae are mutualistically associated with bacteria of the genus *Xenorhabdus*, serving as vectors. After penetrating a host, this complex causes a fast insect pathogenicity and can only be active against a specific host range (Schmutterer & Huber 2005). In many countries they are considered as invertebrate beneficials and no registration is necessary.

The aim of this study was to assess the potential of other biological control agents as alternative or supplemental method to *B.t.*, which are already commercially available on the market as well as non-target and bystander friendly to the greatest possible extent. Therefore, the biological control agents NeemAzal®-T/S and Nemastar® were investigated on their potential to affect larvae of *C. perspectalis* using laboratory bioassays and field trials.

Marerial and Methods

Biological control agents

The plant extract formulation NeemAzal®-T/S (NA), containing 1% of the active ingredient Azadirachtin A, oils and surface-active agents, was kindly provided by the company Trifolio-M GmbH (Lahnau, Germany). The commercial preparation Nemastar®, containing Entomopathogenic nematodes (EPN) of the species *Steinernema carpocapsae*, as well as the wetting agent BREAK-THRU® and an adhesive agent were kindly provided from the company e-nema GmbH (Schwentinental, Germany). The commercially available *Bacillus thuringiensis* (BT) preparations XenTari®, containing the subspecies *aizawai*, and the product Dipel Es®, containing the subspecies *kurstaki*, were kindly donated by Cheminova Deutschland GmbH & Co. KG (Stade, Germany).

For conducting the field trials, treatments were made on-site following recommendations by the producers (Table 12). They were randomly assigned in a split plot design in four replications at the test area (Figure 15). EPN variant incorporated an adjuvant consisting of gel, dispersant and the wetting agent BREAK-THRU®, which was pre-dissolved in ethanol. It was ensured that EPN did not settle to the bottom by constant movement of the suspension in the laboratory and the sprayer in field. Treatments with water served as control. Applications were conducted using pressure sprayers (APV® Akku Roll: 55 l and 7 bar for EPN, filter was removed; Gloria® prima 5 Typ 39 TE: 5 l and 3 bar for NA and BT). Plants were sprayed with defined volumes (Table 12) and it was assured that they were dripping wet.

Plant material

Plants of *Buxus sempervirens* L. var. arborescens L. were purchased from a box tree nursery (Baumschule Atrops, Rheurdt, Germany). A part of the plants were potted and stored in the greenhouse until using for bioassays. Remaining plants had been planted on a testing field for conducting field trials. In addition, cut plant material (twigs of 30 cm length) of the large-leaf variant *B. sempervirens* var. rotundifolia was purchased and stored in a cooling chamber at 10°C until cutting leaf discs for bioassays.

C. perspectalis larvae

C. perspectalis have been reared under laboratory conditions since 2010. Adult moths were kept in rearing cages (length 40 cm × width 36 cm × height 50 cm) with diluted sugar (5%) for feeding. Short box tree twigs (15–20 cm) were regularly introduced into the cages for egg deposition. *C. perspectalis* egg masses (≤ 24h old) were collected from the offered box tree twigs. Hatched larvae were fed on watered box tree twigs in transparent Perspex cylinders (40 cm height × 19.5 cm

diameter) with textile covers. The different larval stages used for the laboratory bioassays (2nd, 3rd and 4th) were determined by the average time of moulting until the particular larval stage, initiated from egg masses of equal age. The duration of larval development was adapted from own previous investigations.

Laboratory bioassays

NeemAzal®-T/S (NA) bioassay with 3rd larval instar

The effect of NA was investigated on 3rd instar larvae by feeding them with treated leaf discs. Therefore, required amount of NA was emulsified with distilled water to prepare different concentrations (0.1%, 0.3% and 0.5%). Treatment with water served as control. Leaf discs were cut from *B. sempervirens* var. Rotundifolia leaves using a cork borer (diameter 1 cm). They were dipped for 30 seconds into the particular emulsions and air dried for 30 minutes. Treated leaf discs were placed in wells (d 3.5 cm) of multiwell plates in which 2 ml water agar (3%) had been filled in to keep the leaf discs from drying. One larva was each added and 20 larvae were used per concentration. Multiwell plates were incubated at 25 °C, 16/8 h L/D, 50% RH. The trial was replicated three times. Larvae were further fed with freshly treated leaf discs two times a week. The vitality and feeding activity were also recorded two times a week. Bioassays were conducted for at least two weeks. Larvae were considered to be dead if they did not respond to prodding with forceps.

Entomopathogenic nematodes (EPN) bioassay with 2nd and 4th larval instars and pupae

Laboratory bioassays were conducted to define the susceptibility of young (2nd instar) and further developed (4th instar) *C. perspectalis* larvae to EPN of the species *S. carpocapsae*. A serial dilution of an EPN suspension was prepared to adjust different EPN concentrations (10, 20, 50, 100 and 200 EPN/ 100 µl). Therefore, product powder containing infective juveniles (IJs) of EPN was diluted in water (2 g in 1000 ml) and well stirred to prepare a stock suspension containing about 200 EPN/ 100 µl. To define the actual EPN concentration and be able to adjust the needed concentrations for the bioassays a dilution and counting procedure was done following the method reported by Glazer and Lewis (2000). Single *Buxus* leaves were treated with 100 µl of EPN suspension and air dried for 15 minutes. Treated leaves were placed in experimental vials (2 x 2 x 2 cm) in which 1 ml water agar (3%) had been filled in to keep the leaves from drying. One larva was added per vial and 15 larvae were included per concentration. Treatment with water served as control. Experimental vials were incubated at 25 °C, 16/8 h L/D, 50% RH. After 3 d, the mortality was determined, food (untreated)

was replenished in the case of survived larvae and vials were incubated for a further 4 days. Thus, the duration of the bioassays was 7 days and the trial was replicated two times per larval stage. Larvae were considered to be dead in the case of a brown coloration of the larvae or if they did not respond to prodding with forceps.

Laboratory bioassays were additionally conducted to define the susceptibility of fresh and matured *C. perspectalis* pupae on EPN of the species *S. carpocapsae*. Tested concentrations (10, 50, 200 EPN/ 100 µl) were prepared as mentioned before. 10 fresh and 10 matured pupae were included per concentration. They were individually placed in experimental vials (diameter 3.8 cm) and directly treated with 100 µl of EPN suspension. Treatment with water served as control. Experimental vials were incubated at 25 °C, 16/8 h L/D, 40% RH. Pupae were daily observed and hatched moths were counted. After 14 d, all non-developed pupae were dissected to examine them for the presence of nematodes.

Field trials comparing commercial preparations (NA, EPN and BT)

Field trial on already infested Buxus hedges at Seligenstadt 2012

In August 2012, a field trial was conducted on already infested *Buxus* hedges in the cloister of a public convent garden in Seligenstadt (50°2'38.75"N 8°58'31.25"E; South-Hessia, Germany) to compare EPN.12.wa, EPN.12.wa+aa with BTa.12 in the field (Table 12). The cloister (Figure 15) consisted of low planted *Buxus* hedges, symmetrically arranged in four rectangles. Before the treatment the current level of infestation was assessed. Therefore eight plots of 0.25 m² (2/ replicate) were defined (Figure 1 a - h) and *C. perspectalis* larvae were searched within this plots and counted. Five days after the treatment (DAT5), living and dead larvae were counted on the total area of treated hedges (about 18 m²) for 10 minutes per replicate to evaluate the mortality directly in field. In addition, treated twigs (5/ replicate) were collected at the application day (DAT0). The 5 samples per replicate, thus 20 per treatment, were cut and transferred to single vials. In the laboratory one *C. perspectalis* larvae obtained from own rearing was added to each vial. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days and mortality was determined.

Field trial on already infested Buxus hedges at Seligenstadt 2013

In June 2013, a second field trial was conducted on already infested box hedges in the cloister (Figure 15) of a public convent garden in Seligenstadt (50°2'38.75"N 8°58'31.25"E; South-Hessia, Germany) to compare NA.13, EPN.13.adj and BTk.13 in the field (Table 12). Before the treatment the current

level of infestation was assessed. Therefore eight plots of 0.25 m² (2/ replicate) were defined (Figure 15 a - h) and *C. perspectalis* larvae were searched within this plots and counted. Seven days after the application, living and dead larvae were counted on the total area of treated hedges for 10 minutes per replicate to evaluate the mortality directly in field. In addition, treated twigs (5/ replicate) were collected three times at DAT0, DAT7 and DAT14. The 5 samples per replicate, thus 20 per treatment, were cut and transferred to single vials. In the laboratory one *C. perspectalis* larvae obtained from own rearing was added to each vial. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days and mortality was determined.

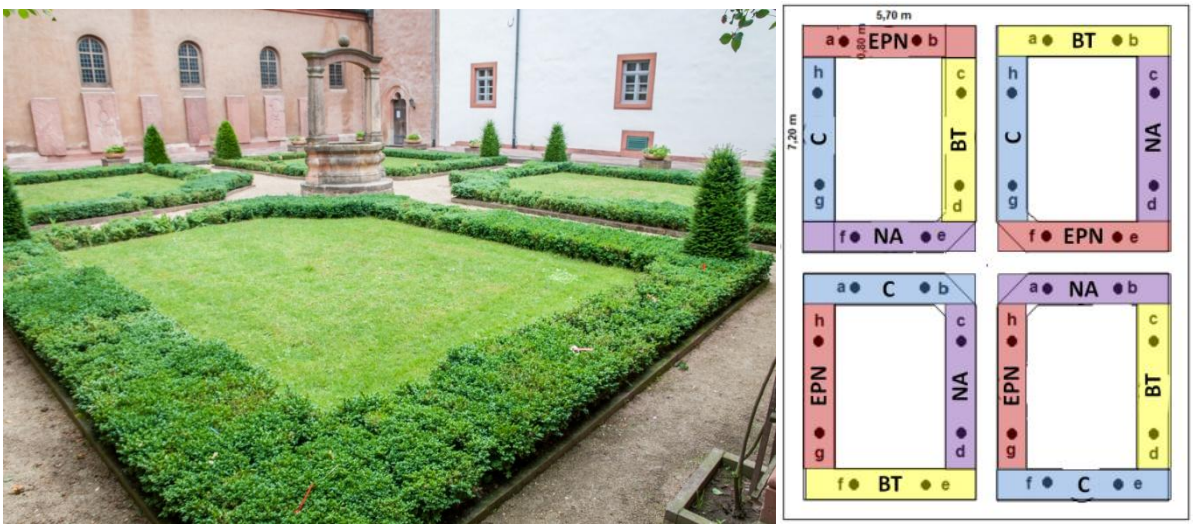


Figure 15 *Buxus* hedges (left) and experimental design (right) of field trials at the cloister in Seligenstadt conducted 2012 and 2013, including four application sites per variant (colored) and defined plots to assess the level of infestation before the application (a – h). Full names of variants see Table 12.

Field trial on intentionally infested Buxus patterns at Darmstadt 2014

In August 2014, a field trial was conducted on *Buxus* plants which were planted on an experimental field in Darmstadt (49°51'53"N 8°40'6"E; Hesse, Germany) to compare NA.14, EPN.14.adj and BTk.14. An experimental area was created, consisting of 16 plots in four lines, wherein one plot comprises four *Buxus* plants (0.25 m²). Each plot was infected with 10 larvae (4^{th} larval instar) obtained from the laboratory. Within 4 days before the application, they were allowed to feed and to produce their typically loose webs in the plant. To minimize the impact of solar irradiation on nematodes, the trial was conducted late in the evening (8:00 pm) as recommend by the producers. Four days after the treatment (DAT4), the deployed larvae were collected from the treated plots. Dead larvae were counted and living larvae were further incubated in the laboratory to determine the cumulative mortality at 3, 7 and 10 days of further incubation (DFI). In addition, samples were

collected four times after the treatment (DAT0, 4, 7 and 11) from the plots to assess the persistence of applicated variants in the field. Therefore, 40 twigs per variant (10 each plot) were cut, transferred into vials and offered to single *C. perspectalis* larvae obtained from the laboratory. Vials were incubated in a climate chamber (25 °C, 16/8 h L/D, 50% RH) for a further 14 days and field mortality of these larvae was determined.

Table 12 Treatments considered in field trials on the biological control of *C. perspectalis*.

Trial	Product (abbreviation)		Content	Concentration	Application rate
2012	Nemastar®	(EPN.12.wa)	*S. carpocapsae*	1.25 million/ l	2 l/ m²
			wetting agent (wa)	0.1 ml / l	
	Nemastar®	(EPN.12.wa+aa)	*S. carpocapsae*	1.25 million/ l	2l/ m²
			wetting agent (wa)	0.1 ml / l	
			adhesive agent (aa)	1.75 g / l	
	XenTari®	(BTa.12)	*B. thuringiensis aizawai*	1 g / l	1 l/ m²
2013	NeemAzal®-T/S	(NA.13)	1% Azadirachtin A	3 ml/ l	0.2 l/ m²
	Nemastar®	(EPN.13.adj)	*S. carpocapsae*	1.25 million/ l	2 l/ m²
			adjuvant*	2 g / l	
	Dipel ES®	(BTk.13)	*B. thuringiensis kurstaki*	1 ml/ l	0.2 l/ m²
2014	NeemAzal®-T/S	(NA.14)	1% Azadirachtin A	5 ml/ l	1 l/ 0.25 m²
	Nemastar®	(EPN.14.adj)	*S. carpocapsae*	2.5 million/ l	1 l/ 0.25 m²
			adjuvant*	2 g / l	
	Dipel ES®	(BTk.14)	*B. thuringiensis kurstaki*	1 ml/ l	0.5 l/ 0.25 m²

* consists of gel, dispersant and wetting agent

Statistical analyses

For statistical analyses the scientific data analysis software Sigma Plot Version 13.0 was used. The effect of treatment and concentration of NA preparations on feeding and mortality as well as the effect of treatment and concentration of EPN preparations on mortality of young and further developed larvae was assessed via One Way ANOVA (α=0.05). If significant, pair wise multiple comparisons were conducted using Tukey tests. To analyse the effect of EPN concentrations on fresh and matured pupae, proportions were compared using a Chi^2-test. Other results are presented in bar graphs, if possible as mean values with standard deviations.

Results

Laboratory trials

Effect of NeemAzal®-T/S (NA) on 3rd larval instars

Investigation on the effect of NA on *C. perspectalis* demonstrated susceptibility of 3rd instar larvae. After 6 days feeding on treated leaf discs, the amount of vital and still feeding larvae was high and ranged between rates of 60% and 80% of exposed larvae (n = 20 / concentration in each trial). No significant impact of treatments on feeding activity could be determined (F = 2.174, df = 3, P = 0.169). Regarding mortality after 6 d, only a few larvae died and no significant impact of treatments was detected (F = 1.065, df = 3, P = 0.416). After 14 d, feeding activity was significantly reduced to the control to less than 10 % in all treatments (F = 202.872, df = 3, P < 0.001). Mortality increased significantly after 14 d of providing leaf discs treated with 0.3% or 0.5% NA (F = 7.318, df = 3, P< 0.05) to a maximum of 62% (Figure 16).

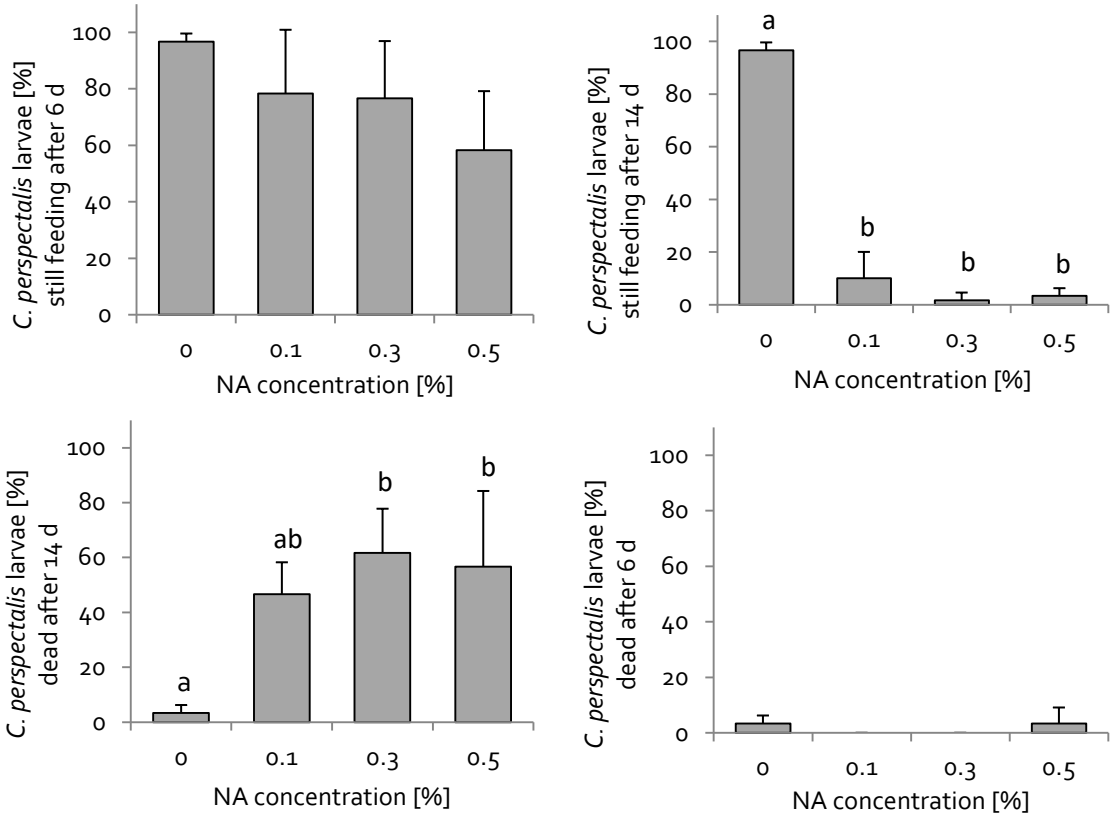


Figure 16 Mean proportion [%] of *C. perspectalis* 3rd instar larvae (n = 20 / concentration) still feeding or dead after 6 and 14 days of providing *Buxus* leaf discs treated with different NeemAzal®-T/S (NA) concentrations (n = 3 trials). Different letters indicate significant differences (ANOVA, Tukey Test, α = 0.05).

EPN bioassays with larvae and pupae

Both tested larval instars (2nd and 4th) were highly susceptible to EPN. After 3 d of exposure mortality was significantly lower for the young larvae ($F = 18.337$, $df = 11$, $P < 0.001$) ranging between 10% and 75% in comparison to further developed larvae were mortality rates ranged between 45% and 100%. The mortality of young larvae was significantly affected after 3 d ($F = 24.279$, $df = 5$, $P < 0.001$) by higher concentrations (100 and 200 EPN / larva, $P < 0.05$), while the 4th instar larvae showed already a significant susceptibility to lower concentrations (20 and 50 EPN/ 100 µl ($F = 12.65$, $df = 5$, $P = 0.004$) at the same time. After 7 days of exposure, mortality was significantly affected by all treatments equal to or higher than 10 EPN / larva regarding both tested larval stages (2nd: $F = 129.672$, $df = 5$, $P < 0.001$; 4th: $F = 85.641$, $df = 5$, $P < 0.001$). A mortality of 100% could be determined after 7 d for all larvae which had been exposed to higher nematode concentrations (50, 100 and 200 EPN/ larva) (Table 13).

EPN were much less effective against *C. perspectalis* pupae. Between 70% and 100% of all treated pupae hatched within 14 days. Only a small amount of the pupae did not hatch (16%), including pupae of the untreated control. There was no significant difference in the proportions of dead fresh and matured pupae and mortality was not related to the tested EPN concentration ($Chi^2 = 1.333$, $df = 2$, $P = 0.513$) (Table 13). Only one matured pupa, treated with 200 EPN contained nematodes, indicating a successful penetration into the insect body. No EPN were detected in the remaining dissected pupae.

Table 13 Mortality [%] (mean ± SD) of young *C. perspectalis* larvae (2nd instar, n = 2 x 15) and further developed larvae (4th instar, n = 2 x 15) after 3 and 7 days exposed to *Buxus* leaves treated with different nematode concentrations [EPN/ larva] (ANOVA, Tukey Test, α=0.05) as well as fresh (f, n = 10) and matured (m, n = 10) pupae once directly treated with different EPN concentrations [EPN/ pupa] (Chi² test, α=0.05). Different letters indicate significant differences between the impacts of concentrations.

[EPN/ larva] [EPN/ pupa]	2nd instar				4th instar				pupae		
	3 d		7 d		3 d		7 d		f	m	
0	0 ± 0	a	3 ± 4.7	a	0 ± 0	a	0 ± 0	a	10	10	a
10	10 ± 14.1	a	70 ± 4.7	b	47 ± 28.3	a	77 ± 14.1	b	10	30	a
20	10 ± 4.7	a	87 ± 9.4	bc	70 ± 14.1	b	97 ± 4.7	b			
50	33 ± 0	a	100 ± 0	c	87 ± 18.9	b	100 ± 0	b	20	20	a
100	70 ± 14.1	b	100 ± 0	c	97 ± 4.7	b	100 ± 0	b			
200	73 ± 9.4	b	100 ± 0	c	100 ± 0	b	100 ± 0	b	20	20	a

Field trials

Field trial on naturally infested Buxus hedges at Seligenstadt 2012

The number of *C. perspectalis* found in the *Buxus* hedges before the application of EPN.12.wa, EPN.12.wa+aa and BTa.12 was in total 191 on 8 m² (32 x 0.25 m² plots), i.e. 24 *C. perspectalis*/ m² (including larvae and a small amount of pupae). 5 d after the application, the estimated number of *C. perspectalis* was 421 including 102 dead larvae on the total area of treated hedges (18 m²), i.e. 23 *C. perspectalis*/ m², including 6 dead larvae/ m². The mean number of dead larvae was high in the BTa.12 treatment where only a few vital larvae were found. In contrast, in the EPN treatments the number of vital larvae was high and only a few dead larvae were found (Figure 17).

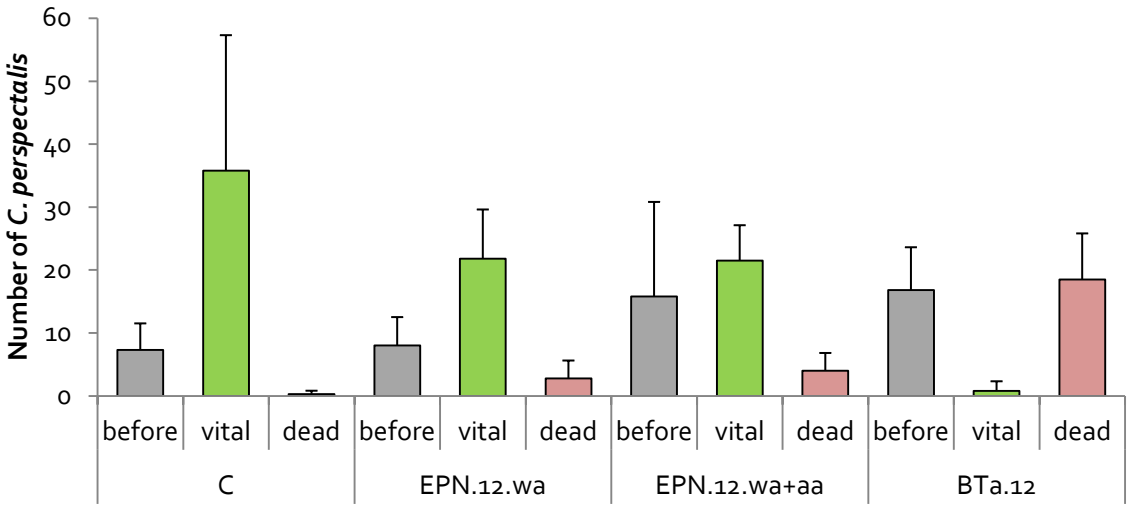


Figure 17 Mean number of *C. perspectalis* found at already infested *Buxus* hedges (n = 4 sites / treatment) before (grey bars) and after (vital: green and dead: red bars) the application of different variants at the cloister at Seligenstadt 2012 (see Figure 15). Full names of tested biological control agents seeTable 12.

The application of EPN.12 and BT.12, caused significant differences in field mortality among the treatments 5 d after the application in 2012 (ANOVA, $F = 81.245$, $df = 3$, $P < 0.001$). BTa.12 (96%) significantly differed from EPN.12.wa (11%), EPN.12.wa+aa (16%) and the control ($P< 0.001$). These rates could be supported in parallel by the mortality rates achieved in the laboratory, after treated twigs were collected at DATo and offered to larvae from the laboratory: EPN.12.wa (55%), EPN.12.wa+aa (80%) and BTa.12 (95%). BT was the most effective agent (Table 14).

Field trial on naturally infested Buxus hedges at Seligenstadt 2013

In total, 60 larvae/ 8 m² (32 x 0.25 m² plots), i.e. 7.5 larvae/ m² were found in the *Buxus* hedges before the application of NA.13, EPN.13.adj and BTk.13 in 2013. 7 d after the application, the counted number of *C. perspectalis* did not differ, but also pupae were found on the total area of treated hedges. The number of *C. perspectalis* found in the different variants did not decrease after the treatment but two dead larvae were found in the EPN.13.adj treated area. In the BTk.13 treated hedges, no larvae or pupae were found after the treatment at all (Figure 18).

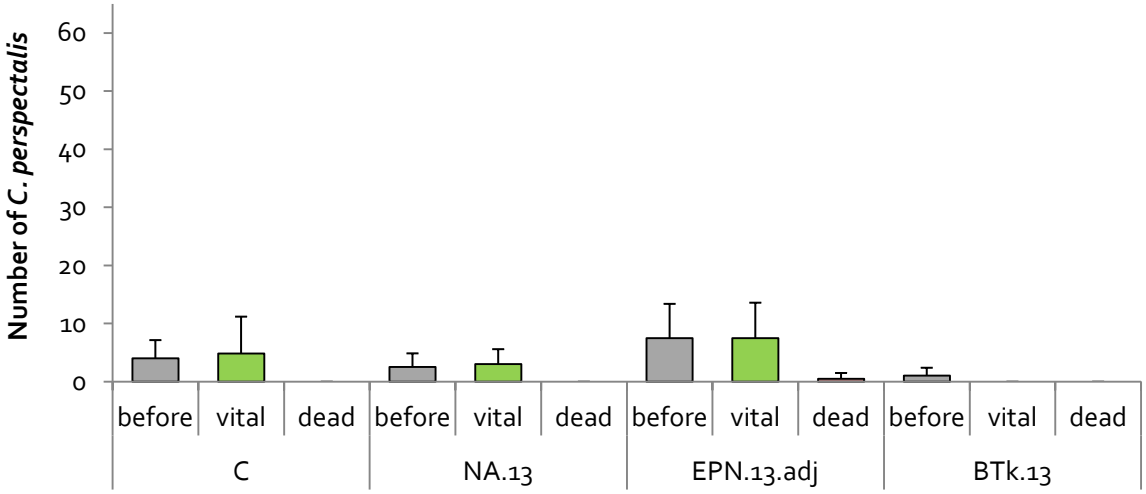


Figure 18 Mean number of *C. perspectalis* found at already infested *Buxus* hedges (n = 4 sites / treatment) before (grey bars) and after (vital: green and dead: red bars) the application of different variants at the cloister at Seligenstadt 2013 (see Figure 15). Full names of tested biological control agents see Table 12.

The persistence in field differed between the variants. Exposure to treated twigs of all tested variants caused dead larvae in the laboratory if they were collected at the application day: NA.13 (30%), EPN.13.adj (55%) and BTk.13 (75%). Larvae exposed to twigs collected on DAT7 and DAT14 with NA.13 or EPN.13.adj were not affected in contrast to the BTk.13 treatment. BT was the most effective agent, and dead larvae were found in 2013 also on DAT7 and DAT14 (10%-15%) (Table 14).

Table 14 Larval mortality [%] of *C. perspectalis* in field as well as after exposure of treated twigs collected at different days after treatment (DAT) and further incubated in the laboratory. Full names of tested biological control agents seeTable 12.

	2012				2013			
	C	**EPN.12.wa**	**EPN.12.wa+aa**	**BTa.12**	**C**	**NA.13**	**EPN.13.adj**	**BTk.13**
field	1	11	16	96	0	0	7	0
DAT0	0	55	80	95	0	30	55	75
DAT7	-	-	-	-	0	0	0	15
DAT14	-	-	-	-	10	0	0	10

Field trial on intentionally infested Buxus patterns at Darmstadt 2014

Not all deposited larvae were retrieved from *Buxus* plots four days after the treatment (Figure 19, numbers in parentheses). At this day (DAT4) 66% of retrieved larvae, which were exposed to BTk.14 and 30% of larvae exposed to EPN.14.adj were dead. In contrast, all collected larvae from C and NA.14 were alive. Mortality rates of the field collected larvae further increased with the days of further incubation (DFI3, 7 and 10) of the survived larvae in the laboratory. Thus, the mortality of BTk.14 treated larvae rose to 100% at DFI3. Mortality of EPN.14.adj treated larvae reached the maximum of 63% at DFI3. In addition larvae treated with NA.14 showed an increased mortality, which reached 54% at DFI10 (Figure 19). These results suggest that larvae already have been affected in field.

In addition, the persistence in field differed between the variants. When exposing larvae to twigs collected at DAT0, 93% of EPN.14.adj treated larvae and 78% of BTk.14 treated larvae died. BTk.14 was the most persistent variant and caused high mortality rates (>90%) of larvae exposed to twigs collected at DAT4 and 11. The persistence of EPN.14.adj fluctuated and rapidly decreased in field, but after seven days larvae were infected by nematodes again as conferred by dissection. In contrast, larvae exposed to twigs treated with water or NA.14 were not affected (Figure 20).

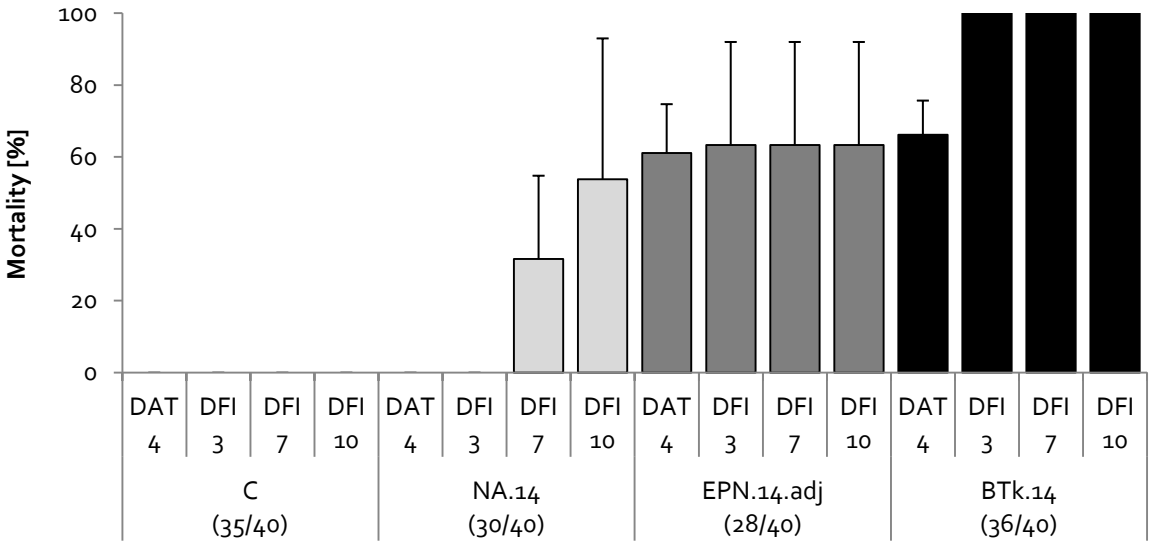


Figure 19 Cumulative mortality [%] of retrieved *C. perspectalis* larvae, collected 2014 from *Buxus* plots in field (n = 4) four days after treatment (DAT). Mortality was assessed after 3, 7 and 10 days of further incubation (DFI). Numbers in parentheses show number of larvae (retrieved / deployed). Full names of tested biological control agents see Table 12.

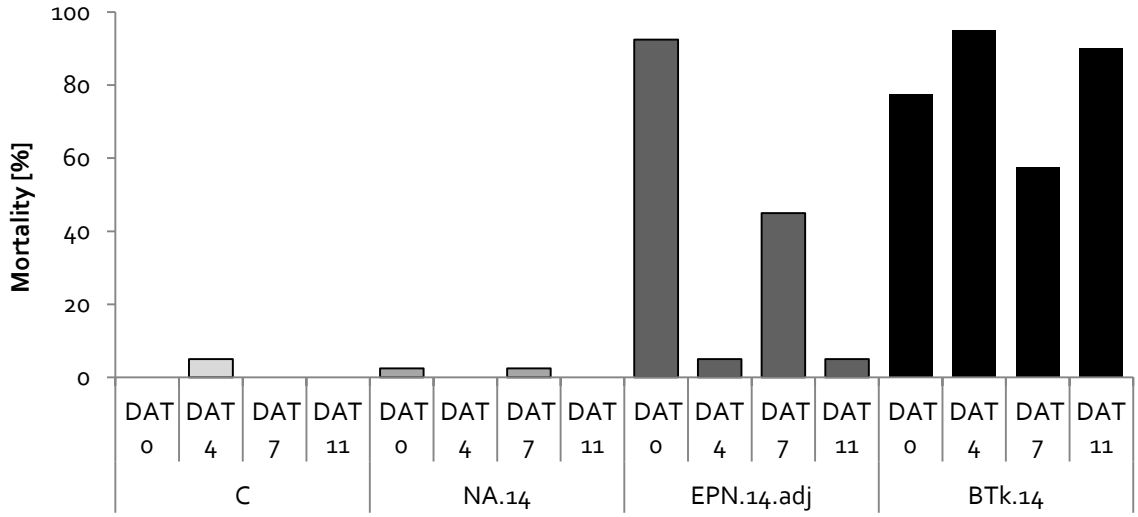


Figure 20 Persistence of potential biological control agents in the field. Mortality [%] of *C. perspectalis* larvae (n = 40 / treatment) fed with treated twigs collected 2014 at 0, 4, 7 and 11 days after treatment (DAT) of different variants in the field. Full names of tested biological control agents see Table 12.

Discussion

This is the first report on the susceptibility of European *C. perspectalis* populations to the biological control agents Nemastar® (EPN) and NeemAzal®-T/S (NA). The results demonstrate clearly the potential of both tested agents to control *C. perspectalis*, but also the challenges of application on *Buxus* plants in the field.

The vulnerability of *C. perspectalis* larvae (3rd instar) to NA was assessed by the potential impact on feeding activity and survival of larvae after repeated ingestion of treated leaf discs. In a preliminary trial the impact on young (2nd instar) and further developed (4th instar) larvae was tested based on uniquely ingestion of single treated *Buxus* leaves. The responding time was highly extended and larvae were starving many weeks until all of them died (up to seven weeks). Only minor differences occurred in the reaction of the tested larval stages (Table 13). In the present study, the method of investigation was improved to a more realistic situation. 3rd instar larvae had been fed continuously with freshly treated leaf discs. There was only little variation in the response on the different tested NA concentrations (1-5 ml/ l). None of the tested concentrations led to mortality earlier than 6 days after first ingestion of treated leaf discs. This kind of time delay is well known regarding investigations on the impact of NA to several pests. Most insect pests continue to feed on the treated plants for some time, but as a rule, the amount of food ingested by them is considerably reduced, owing to the influence of the "secondary" anti-feedant effect (Schmutterer 1990). For example Efil et al. (2005) noted a duration of 6 days until larval mortality occurred as well, when leaf discs had been treated with concentrations of 0.5-2.5 ml/ l before offering them to larvae of the Beet armyworm *Spotoptera exiqua* Hübner (Lepidoptera: Noctuidae). Application rates used in our study complied with the usual applications of 1-7 ml/ l NA for bioassays and recommended rates of 250 ml/100 l water against lepidopteran pests in the field (Dammini Premachandra et al. 2005; Erler et al. 2010). It can be concluded that *C. perspectalis* larvae are susceptible to ingested NA at common concentrations (preferably 3-5 ml/ l), causing feeding stop and larval mortality within two weeks in laboratory bioassays.

Table 15 Duration [days] until > 50% *C. perspectalis* larvae (mean ± SD) of different larval stages (2nd and 4th) stopped feeding and died after unique ingestion of one *Buxus* leaf treated with different concentrations of NeemAzal-T/S.

		0.1%	0.3%	0.5%	1.0%
feeding stop	2nd	17.5 ± 0.7	12.0 ± 2.8	12.0 ± 2.8	10.5 ± 0.7
	4th	15.0 ± 4.2	12.0 -	12.0 -	12.0 -
mortality	2nd	29.5 ± 6.4	21.0 ± 5.7	17.5 ± 0.7	17.5 ± 0.7
	4th	30.0 ± 4.2	27.0 -	22.0 ± 1.4	18.0 -

The presented results suggest that also EPN affected *C. perspectalis* larvae. In preliminary own investigations, three EPN species were examined on their impact on *C. perspectalis* larvae. The species *Heterorhabditis bacteriophora* showed the lowest effect on larval mortality (8%-15%) in comparison to *S. feltiae* (46%-100%) and *S. carpocapsae* (85%-100%) at concentrations of 25-200 EPN/ larva (Göttig 2012). Shannag and Capinera (1995) compared five EPN species against the Melonworm moth *Diaphania hyalinata* Linnaeus (Crambidae), a close relative to *C. perspectalis,* and determined highest infectiousness of *S. carpocapsae* as well. Furthermore, *D. hyalinata* was significantly less susceptible at first instar larval stage and pupae than older larvae, which was also evident in this study. In contrast, Choo et al. (1991a) reported that there was no difference in infection on Japanese *C. perspectalis* larvae by *H. bacteriophora* and *S. carpocapsae* achieving 100% mortality in all treatments ranging between 10 and 80 EPN/ larva. Our investigation on the infectivity of different *S. carpocapsae* concentrations (10-200 EPN) on *C. perspectalis* demonstrated a high susceptibility of both tested larval instars (2nd instar: 10%-75% and 4th instar: 45%-100%). Infection of further developed larvae was faster in comparison to young larvae, regarding the pathogenicity after 3 d. It is known that insect size as well as nematode size can influence the potential of EPN species to invade potential host species, because they are most frequently penetrated via body orifices (Bastidas et al. 2014). Further developed larvae have bigger orifices or probably fed more of the leaves or had more contact to the surface. It was found that pupae were less vulnerable to EPN, even if adult nematodes were found in one of 13 dead and dissected pupae in our study. This could be observed in the case of treated winter cocoons of *C. perspectalis* larvae (usually 3rd instar larvae), too (data not shown). In contrast, infection of cocooned Codling moth larvae (*Cydia pomonella* L., Lepidoptera: Tortricidae) was high and mortality ranged between 30% and 100% after application of *S. carpocapsae* (0.5–5.0 million/ tree) in the field (Unruh & Lacey 2001).

Although both biological control agents can affect *C. perspectalis* in the laboratory, limited success was achieved in field applications. In the presented field trial on naturally infested *Buxus* hedges

(2012 and 2013), it was not possible to detect an exact difference in the occurring amount of *C. perspectalis* before and after the treatment. In general, the method of feeding treated leaf samples to laboratory reared larvae proved to be reliable in order to quantify effects even in field trials. In addition, field studies conducted on *Buxus* hedges intentionally infested with a defined number of larvae (2014) facilitated the quantitative recording of the effect. Thus, the method was more suitable to assess field mortality.

In this study, the particular order of the effectiveness of NA, EPN and BT was comparable in each experiment. The application of NA resulted in low response of treated larvae and larval mortality constantly took a long time. This extensive duration until the larvae stopped feeding and died could be problematic for rapid efficiency in acute damage cases. But NA may be considered for applications on small larvae as already mentioned for other targets, in the case that feeding damage temporary does not endanger the plants health. Application would be of great advantage if other sucking pests are present on the *Buxus* plant or being part of applications combining more than one control agent. Impacts were proofed on over 400 insect species, especially on lepidopteran species (> 138) (Schmutterer & Huber 2005).

Using EPN in field produced mortality rates about 16-65% at a range of 2.5 million/ m^2 but high fluctuations were observed in persistence tests. Mortality rose up to 80%, if field treated twigs collected at the application day were fed to larvae in the laboratory. Field mortality was in the same range as results of Shannag and Capinera (1995) where infection rates of 52%-55% were produced in field applications of 5 billion EPN per hectare (0.5 million/ m^2) on *D. hyalinata* on crookneck squash foliage. As beneficial agent, the application of EPN requires no permission and there are no user restrictions in many countries. The rate of mortality in field and nematode success to penetrate host species is strongly related to weather conditions, the application technique and easy accessibility of the host on the plant foliage, in contrast to soil applications. Factors like the UV irradiation and temperature, as well as the relative humidity play a decisive role in foliar application and the persistence of EPN in field (Glazer 1992; Smits 1996; Arthurs et al. 2004). Many advantages also apply to EPN. They are partially able to actively seek their host, they are almost harmless to non-target organisms, are easily mass produced, have a broad host range and have the ability to kill their hosts rapidly (Shannag & Capinera 1995; Kaya & Gaugler 1998).

Because larvae of *C. perspectalis* are highly susceptible to BTk and BTa, the use of plant protection products based on *Bacillus thuringiensis* is currently the only alternative to chemical insecticides and available effective biological control method. However, requirements for proper application are high

and pesticide resistance is becoming an increasingly important factor in the selection of suitable control agents, even in the case of using BT against other lepidopteran species (Heckel et al. 2007; Kang et al. 2014). Resistance can change rapidly the usable insecticides arsenal. Therefore, plant-derived natural products like NA as well as beneficials like EPN are considered to be valuable candidates to prevent resistance on common pesticides.

In conclusion, there is a higher variability in terms of the effects of the investigated agents Nemastar® and NeemAzal®-T/S compared to the constant effectiveness of Dipel ES® in the field. But their use would be possible if feeding damage and proper surveillance of the result will be considered in order to be capable of repeating the application timely. More frequent and timely applied treatments may improve efficacy and a combination with further measures ought to take place.

Chapter V. GÖTTIG S., KORN S. & HERZ A. *Archives of Phytopathology and Plant Protection* (submitted)

Repellent and toxic properties of plant oils and extracts on *Cydalima perspectalis* Walker (Lepidoptera: Crambidae)

Submitted on 17.05.17

S. Korn supported the laboratory investigetions on the effect of *Thymus vulgaris* L. and *Melaleuca alternifolia* M. & B. on *C. perspectalis* larvae in the framework of a master thesis, conducted at the JKI Darmstadt (Korn 2016).

Abstract

Buxus plants in Europe become frequently infested by the Box tree moth, *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) but applicable biological control methods are limited. To make a first step in preventing egg deposition on *Buxus* plants, seven essential oils (*Carum carvi*, *Juniperus communis*, *Lavendula angustifolia*, *Melaleuca alternifolia*, *Pinus sylvestris*, *Rosmarinus officinalis* and *Thymus vulgaris*), one seed oil (*Sambucus nigra*) and six plant extracts (*Equisetum arvense*, *Juniperus communis*, *Quassia amara*, *Salvia officinalis*, *Tanacetum vulgare* and *Thymus vulgaris*) were investigated on their potential to influence oviposition preference in the greenhouse. The four essential oils exhibiting the highest repellency were further assessed in laboratory leaf disc bioassays on larvae. Despite the strong repellent effect of *S. nigra* on female oviposition, no impact could be detected on the larvae. These were significantly susceptible to exposed leaf discs treated with *T. vulgaris* and *M. alternifolia* essential oils including feeding deterrence, survival and larval weight. *T. vulgaris* was the most effective oil considering both effects. The effective lethal concentration (LC_{50}) of *T. vulgaris* was calculated to be 1% at 72 h. Our results suggest the possibility of using essential oils to protect *Buxus* plants in order to reduce the overall risk for defoliation by larval feeding.

Keywords: Box tree moth, biological control, egg deposition, feeding deterrence, toxicity, *Thymus vulgaris*, *Sambucus nigra*

Introduction

The herbivorous Box tree moth *Cydalima perspectalis* Linnaeus (Walker 1859) (Lepidoptera: Crambidae) has been identified as the most important pest of plants of the genus *Buxus*. It is a natural pest in its native range China, Japan, Korea and India (van der Straten & Muus 2010; Wan et al. 2014). Since its introduction into Europe in 2007 (Billen 2007; Leuthardt et al. 2010) it became highly invasive and destructive on ornamental trees and natural stands of *Buxus*, frequently leading to complete defoliation. In Germany, *C. perspectalis* is a well-established bivoltine species (Göttig & Herz 2017) and perceived as monophagous on *Buxus* plants. In Asia, ten common *Buxus* species have been reported as hosts (Wan et al. 2014) but it was additionally found damaging plants of other genus like *Murraya* (*Murraya paniculata* (L.) Jack) in China (Wang 2008). Also plant species of the genus *Euonymus* (*Euonymus japonicus* Thunb. and *Euonymus alatus* Thunb.) and *Ilex* (*Ilex purpurea* Hassk.) were mentioned as supplemental host in Japanese literature (Shi & Hu 2007). But these assumptions could not be confirmed for Europe yet, despite several investigations and field observations. Unpublished tests from the Plant Protection Service in the Netherlands (van der Straten & Muus 2010) and investigations on potential host plants belonging to the family Buxaceae (*Pachysandra terminalis* Michx. and *Sarcococca* sp. Lindl.) and *Ilex* (*Ilex crenata* Thunb.) in Germany (Zimmermann 2014) as well as field observations in Turkey (Hizal et al. 2012) suggest, that there are no other host species than *Buxus* sp. in Europe. At the same time, a selective behavior of females in oviposition could be observed in different studies, concerning variants within the genus *Buxus*. Leuthardt and Baur (2013) tested the oviposition preference of *C. perspectalis* females on five *Buxus* varieties frequently planted in Switzerland and Europe. They found a preference of female moths for laying their egg masses on the large-leaved variety *B. sempervirens* var. 'Rotundifolia' L., while other small-leaved species (*B. microphylla* var. 'Faulkner' Siebold & Zucc.) were less frequently considered. In contrast, Maruyama (1992) concluded based on investigations on injury levels that *B. microphylla* was the preferred host plant for Japanese *C. perspectalis* populations.

The selective behavior of adult lepidopteran females to prefer a potential host plant for oviposition, may be explained by the relationship between oviposition preference and the offspring survival and performance, being part of the complex evolutionary ecology of host plant associations in herbivorous insects (Thompson 1988; Thompson & Pellmyr 1991; Awmack & Leather 2002). This is an important mechanism for understanding herbivorous insect attacks on host plants because the selection connects expected food quality with offspring performance and the probability of their survival (Zhang et al. 2012). Thus, in theory adult females tend to lay more eggs on plants conductive to offspring performance. Gripenberg et al. (2010) assessed in a meta-analysis the

balance of evidence for and against this preference–performance hypothesis (PPH) where insects will oviposit on hosts on which their offspring fare best. Despite the occurrence of counterexamples, they concluded a clear positive association. Moreover they pointed out that female preference for good quality plants was stronger in monophagous than in polyphagous insects. Further, it is well known that sensory mechanisms are essential for lepidopteran females to recognise their hosts and reject unsuitable plants. The sensory cues that elicit or inhibit oviposition clearly play an important role in the survival of most herbivorous insects because the hatching larvae are often restricted in mobility. Chemicals are responsible for mediating many of the behavioral steps. The sensory cues play a critical role in host selection, since the final signal for oviposition or rejection usually depends on contact chemoreception of stimulants or deterrents (Renwick & Radke 1988; Renwick 1994). Thus, selective behavior of herbivorous lepidopteran females can be influenced by a lack of stimulants as well as the presence of deterrents.

In the 1970s, the possibility of using deterrents and repellents as crop protectants became attractive. In the 1990s the fumigant and contact insecticidal activities of essential oils became evident and the development of oil-based insecticides, fungicides and herbicides for agricultural and industrial applications has considerably raised interest (Isman 2006). Based on a recent literature review the proportion of all journal papers on insecticides dealing specifically with botanicals has risen from less than 2% in 1980 to over 21% in 2011, including many investigations on essential oils (Isman 2015). Essential oils are secondary plant metabolites produced in plant species belonging mostly to a few families (Asteraceae, Lamiaceae, Lauraceae and Myrtaceae). They are stored in various plant organs like flowers, leaves, wood, roots, rhizomes, fruits and seeds (Regnault-Roger et al. 2012) and mainly represent the bioactive fraction of plant extracts, occurring in various proportions. The chemical composition of essential oils is very complex and can contain about 20-60 components. Two major groups of different biosynthetic origin occur, aromatic or aliphatic constituents and terpenes or terpenoids, mostly monoterpenes, phenols and sesquiterpenes. Essential oils are characterised by two or three major components which determine the biological properties (Bakkali et al. 2008). Essential oils can have a multitude of biological effects, particularly as repellent or attractant and play an important role in natural defense and protection of plants. Their beneficial effects like the utility as antibacterials, antifungals, antivirals and insecticides are due to prooxidant effects on the cellular level, they can show cytotoxic effects on living cells (Bakkali et al. 2008).

This study was addressed to find out an approach for an environmentally and user friendly control method to prevent *Buxus* plants from *C. perspectalis* egg deposition and larval feeding, thus contributing to an advance for the avoidance or reduction of required insecticides. As a strictly

monophagous herbivorous species, *C. perspectalis* reacts especially on physicochemical properties of *Buxus* plants. We conducted our experiments in order to manipulate these properties and provide answers to the following questions: (i) do the application of plant substances deter females from oviposition; (ii) do essential oils with potential repellent properties additionally affect *C. perspectalis* larvae and if it is the case (iii) does a correlation between essential oil concentration and larval response (mortality) occur. The aim was to find a repellent substance with toxic properties, which could serve to reduce the overall risk for defoliation of *Buxus* plants by larval feeding. Plant extracts were examined because of their advantages regarding the preparation and application, especially for non-professional users.

Marerial and Methods

C. perspectalis moths and larvae

C. perspectalis have been reared under laboratory conditions since 2010. Adult moths were kept in rearing cages (length 40 cm × width 36 cm × height 50 cm) with diluted sugar (5%) for feeding. Short box tree twigs (*B. sempervirens* L.) were regularly introduced into the cages for egg deposition. *Buxus* plant material was obtained from a box tree nursery (Anzuchtbaumschule Atrops, North Rhine-Westphalia, Germany). *C. perspectalis* egg masses (≤ 24h old) were collected from the offered box tree twigs. Hatched larvae were fed on watered box tree twigs in transparent Perspex cylinders (40 cm height × 19.5 cm diameter) with textile covers. For toxicity tests in the laboratory, third instar larvae (L3) were used. The larval stage was determined by the average time of moulting until the particular larval stage, emanated from egg masses of equal age. The duration of larval development was adapted from own previous investigations. For the studies on oviposition, adult moths were used, freshly hatched and mated (≤ 72 h old) during the weekend before testing.

Plant oils and extracts

A total of 14 variants were used (Table 16). The product Quassia-MD was kindly obtained from Trifolio-M GmbH (Dr.-Hans-Wilhelmi-Weg 1, 35633 Lahnau, Germany) and prepared as mentioned by the producer (0.02 g powder mixed with 1 l distilled water). Botanical material for cold water extracts was purchased as dried plant powder from the company F. Schacht GmbH (Bültenweg 48, 38106 Braunschweig, Germany). The method of cold water extracting was chosen because certain active ingredients should not be destroyed by using hot water. Extracts were prepared as mentioned by the producer. Plant powder (2.5 g) was mixed with distilled water (0.5 l) and the extraction was performed 24 h at 8°C in opened SCHOTT DURAN® glass bottles. The rough and fine particles were

filtered in two steps and filled in an atomiser (50 ml). Essential oils, originating in different countries, were purchased from Dagmar Köhler- Baccara Rose (Weseler Str. 2, 46519 Alpen, Germany) and used in different concentrations (between 0.5% and 5% v/v) as been required for the various examinations. The pine oil was provided by courtesy of the company German Pellets, Verfahrenszentrum Reichstädt (VZR) GmbH (Am Hofbusch 4, 01744 Dippoldiswalde, Germany). The cold pressed elder seed oil was produced and kindly provided by the research laboratory Dr. Wolfram Radig (Sophienstr. 15, 99518 Bad Sulza, Germany). Essential oils and the elder seed oil were diluted with distilled water and well shook before used. For conducting the leaf disc trials in the laboratory, Tween20 (1%) was added to emulsify the suspension.

Table 16 Plant extracts and essential oils, which had been investigated on their repellent effect on oviposition preference of adult *C. perspectalis* females in the present study.

Plant extracts	**species**	**family**	**main constituents**
Quassia	*Quassia amara* L.	Simaroubaceae	Quassin, Neoquasssin[a]
Horsetail	*Equisetum arvense* L.	Equisetaceae	Flavonoids, Ester, Silicic acid[b]
Tansy	*Tanacetum vulgare* L.	Asteraceae	Camphor, α-Thujon, Borneol[c]
Sage	*Salvia officinalis* L.	Lamiaceae	α-Thujon, Camphor, Ketone[c]
Thyme	*Thymus vulgaris* L.	Lamiaceae	Thymol, p-Cymene, γ-Terpinene[c]
Juniper	*Juniperus communis* L.	Cupressaceae	α-Pinen, Limonene, δ-3-Carene[c]
Essential oils			
Thyme	*Thymus vulgaris* L.	Lamiaceae	Thymol, p-Cymene, γ-Terpinene[c]
Lavender	*Lavendula angustifolia* Mill.	Lamiaceae	Linalool, tau-Cadinol, Myrcene[c]
Tea tree	*Melaleuca alternifolia* M.& B.	Myrtaceae	Terpinen-4-ol, α and γ-Terpinene[c]
Rosemary	*Rosmarinus officinalis* L.	Lamiaceae	1,8-Cineole, α-Pinen, Camphor[c]
Juniper	*Juniperus communis* L.	Cupressaceae	α-Pinen, Limonene, δ-3-Carene[c]
Caraway	*Carum carvi* L.	Apiaceae	Linalool, Camphor, γ-Terpinene[c]
Pine	*Pinus sylvestris* L.	Pinaceae	α- Pinen, δ-3-Carene, δ-Cadinene[c]
Seed oil			
Elder	*Sambucus nigra* L.	Adoxaceae	Asparagine, Serine, Glutamine[d]

[a] producer information, [b] Marchand P.A. (2012), [c] Kumari et al. (2014), [d] Peumans et al. (1991)

Repellent effect on the oviposition of adult C. perspectalis females

In this study, seven essential oils, one seed oil and six plant extracts of various families (Table 16) were investigated to determine potential repellent properties on egg deposition of *C. perspectalis* females. Two-choice experiments in cages (BugDorm 57 x 57 x 57 cm) were conducted in the greenhouse (25°C ± 5°C, 40% RH, daylight during summer months, additionally lighted during winter months, 16:8 light/dark). Two flasks with watered *Buxus* twigs (treated and non-treated) of the same size and texture (15-20 cm) were offered per cage to one *C. perspectalis* pair (≤ 72 h old),

consisting of a female and a male. Oils (5% v/v) and extract preparations had been sprayed 10 times (approx. 3 ml) with an atomiser (50 ml) on the *Buxus* twigs. Twigs were air dried for 30 minutes and placed with maximum distance to each other in the cage. A sugar solution (5%) was further added in the centre to provide a feeding source. In one trial, three cages per treatment were set up and three treatments were tested simultaneously. Cages were arranged in blocks of three, each containing the three concurrently tested variants. Flasks were assigned to the pairs always randomly. The number of deposited eggs was recorded and twigs were replaced and freshly treated daily. Since there were large fluctuations in regards of starting and the extent of the oviposition, the deposited eggs were summarized per variant and trial and had to be at least 100, otherwise the trial was repeated. If dead females occurred, they were replaced immediately. The experimental duration was five days and three replicates were conducted per trial.

Effects of essential oils on the feeding deterrence, survival and weight of C. perspectalis larvae in laboratory leaf disc bioassays

The four most effective essential oils which exhibited the highest repellent effect on the oviposition of *C. perspectalis* females were selected to evaluate the toxicity to third instar larvae. Plant extracts could not be considered in the investigations on toxicity because cold water extracts as used in our trials cannot provide exact indication of the present concentrations. Therefore, a bioassay was conducted with treated *Buxus* leaf discs, considering two concentrations of essential oils (1% and 5% v/v) and taking into account the feeding deterrence, survival and weight of the larvae. Leaf discs were made from single leaves of the large-leaved variety *B. sempervirens* 'Rotundifolia', using a cork borer (diameter 1.5 cm). Cut discs were dipped for 120 seconds into the particular variant emulsions and air dried for 30 minutes on filter paper. They were placed individually in experimental vials (2 x 2 x 2 cm) in which 1.5 ml water agar (3%) had been filled in to keep the leaf discs from drying. One larva was added to each cube. Two control variants were included: Leaf discs dipped in distilled water and additionally in distilled water with added Tween20 (1%) to make sure that it had no effect on the feeding larvae. For each trial, 15 larvae per variant were deployed and three replicates were conducted in a climate chamber (25°C ± 1°C, 60% RH, 16:8 light/dark). Survival and feeding deterrence were determined after 24 h, 48 h and 72 h. Larvae were considered to be dead if they did not respond to prodding with forceps. The feeding damage was evaluated by visual estimating leaf disc consumption and integrating in six feeding classes (1 = 0%, 2 = 0-25%, 3 = 25-50%, 4 = 50-75%, 5 = 75-100% and 6 = 100%). Additionally, the weight of outlived larvae was determined once after 72 h at the end of the trial.

Concentration response curve and lethal concentration (LC_{50})

The most effective oil, including feeding deterrence, survival and weight of *C. perspectalis* larvae was selected to define the effective lethal concentration (LC_{50}) by establishing a concentration response curve. Therefore, bioassays were conducted (as described above) determining the mortality of *C. perspectalis* larvae after offering leaf discs treated with different concentrations of the selected oil (0.5%, 1%, 2.5% and 5% v/v). For each trial, 10 larvae were deployed per concentration. Four replicates were conducted in a climate chamber (25°C ± 1°C, 60% RH, 16:8 light/dark). Mortality was determined after 24 h, 48 h and 72 h.

Statistical analyses

Statistical analyses was done with RStudio Version 01.0.136 (RStudio 2016). To analyse the proportions of deposited eggs a 2-sample chi²-test for equality of proportions with continuity correction was done to see whether mean proportions of eggs deposited on treated and non-treated twigs per variant differ significantly from an equal distribution of 50%. Because the number of deposited eggs per trial differed between 200 and 1400, a Repellency Value [RV = number of eggs deposited on untreated twigs / (number of eggs deposited on the untreated twigs + number of eggs deposited on the treated twigs)] was calculated (Mauchline et al. 2005; Daniel 2014). To compare variants the calculated RV were arcsin transformed and an ANOVA was performed ($F = 3.676$, $df = 16$, $P = 0.01$) followed by a post-hoc TukeyHSD test ($\alpha = 0.005$). To analyse the toxic effects of oils on larval feeding activity, survival and weight, t-tests were performed after passing Shapiro-Wilk normality test and Brown-Forsythe equal variance test. The program ToxRat Std Version 2.10 (ToxRat 2010) was used to evaluate the effect of applied oil concentrations (log transformed) on larval mortality after 72 h. A chi²-test (df = 2) for goodness of fit was performed. Control mortality was compensated using Abbott's formula (Abbott 1925). The LC_{50} value was calculated based on a probit analysis using linear maximum likelihood regression.

Results

Repellent effect on the oviposition of adult C. perspectalis females

Females deposited eggs in different proportions on untreated and treated twigs per variant in the cage tests (Figure 21). There was a significant impact of variants on the choice of female egg deposition ($F = 3.43$; $df = 13, 28$; $P = 0.003$). Mean proportion of eggs differed significantly from an equal egg distribution of 50% as indicated by RV-values ≥ 0.63 in the variants: *S. nigra* seed oil (RV = 0.94, $\chi^2 = 45.86$, $df = 1$, $P < 0.001$), *T. vulgaris* plant extract (RV = 0.78, $\chi^2 = 15.82$, $df = 1$, $P < 0.001$), *T.*

vulgaris essential oil (RV = 0.65, χ^2 = 4.01, df = 1, P < 0.05), *S. officinalis* plant extract (RV = 0.65, χ^2 = 4.01, df = 1, P < 0.05) and *P. sylvestris* essential oil (RV = 0.63, χ^2 = 2.93, df = 1, P < 0.1). In contrast, females deposited more eggs on treated twigs in the variants *T. vulgare* (RV = 0.48), *E. arvense* (RV = 0.47), *R. officinalis* (RV = 0.46), *Q. amara* (RV = 0.43) and *C. carvi* (RV = 0.39), indicating no repellent effect on egg deposition.

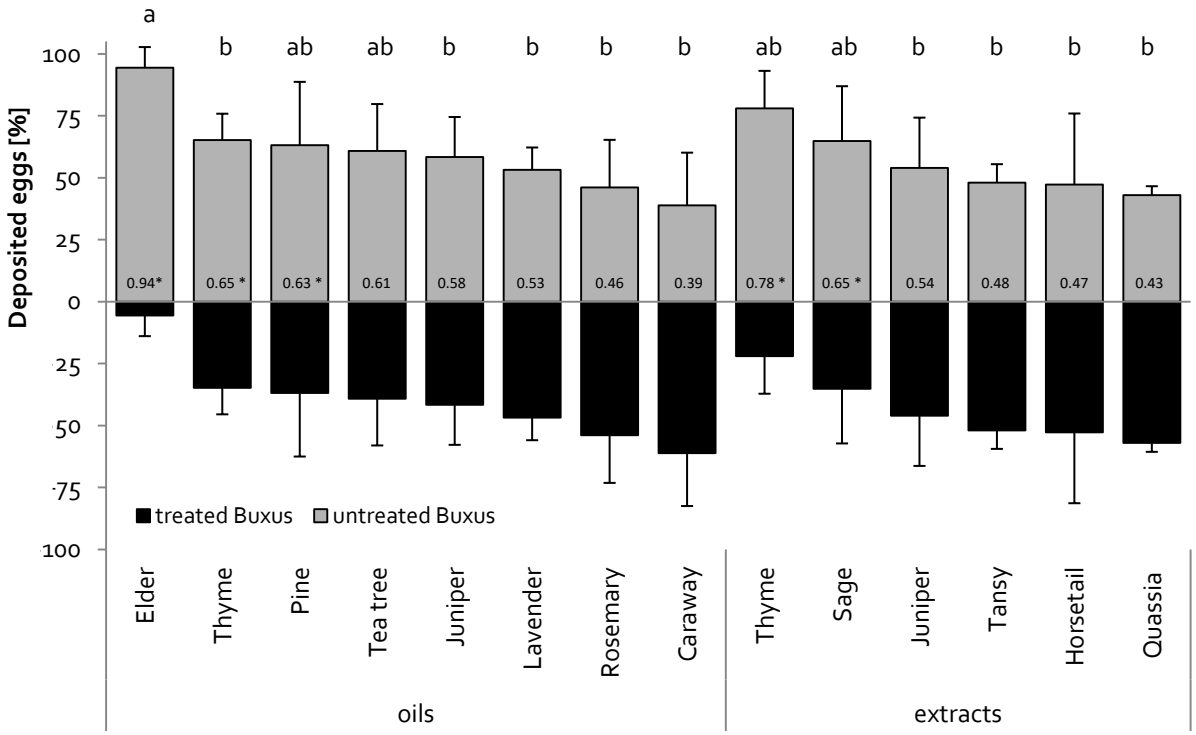


Figure 21 Mean proportion [%] of eggs deposited on untreated and treated twigs. The values given in the bars correspond to the Repellency Values (RV). Asterisks show significant differences from an equal egg distribution of 50% (chi²-test). Letters indicate significant differences of arcsin transformed RV-values (ANOVA, TukeyHSD test, α = 0.005).

Effects of oils on the feeding deterrence, survival and weight of C. perspectalis larvae in laboratory leaf disc bioassays

S. nigra, *T. vulgaris*, *M. alternifolia* and *P. sylvestris* were the most repellent oils determined by oviposition preference tests in the greenhouse and these were also tested for their impact on larvae. Despite the strong repellent effect of *S. nigra* seed oil on the oviposition of *C. perspectalis* females, no toxic impact could be detected in laboratory bioassays within 72 h. High feeding activity (mean feeding class 6 = 100% leaf disc consumption) and no mortality or weight reduction of larvae were recognized in comparison to the control. In addition, treatments with *P. sylvestris* or the additive Tween20 had no impact. Only two of the four selected essential oils produced significant effects on the performance of *C. perspectalis* larvae. *M. alternifolia* significantly influenced the feeding activity

(t = 6.9, df = 4, p = 0.002), survival (t = 3.5, df = 4, p < 0.05) and larval weight (t = 7, df = 4, p = 0.002) after ingestion parts of leaf discs treated with higher concentration (5%). The feeding activity was reduced (mean feeding class 2 ≤ 25% leaf disc consumption) but more than 50% of the larvae survived 72 h of exposion. *T. vulgaris* affected larval performance at both tested concentrations (1% and 5%). Less larvae survived (1%: t = 3.5, df = 4, p < 0.05; 5%: t = 24.9, df = 4, p < 0.001), their weight was reduced (1%: t = 4.9, df = 4, p = 0.008; 5%: t = 6.3, df = 4, p = 0.003) and their feeding activity was totally deterred (mean feeding class 1 = 0% leaf disc consumption; p < 0.001, t-value was infinity high and could not be determined exactly because all values of tested groups were equal (= 1), but clearly significant different from control). Thus, treatment with *T. vulgaris* was most effective in comparison to the application of all other tested substances. First effects were observed already after 24 h and only a mean of 7% survived (Figure 22).

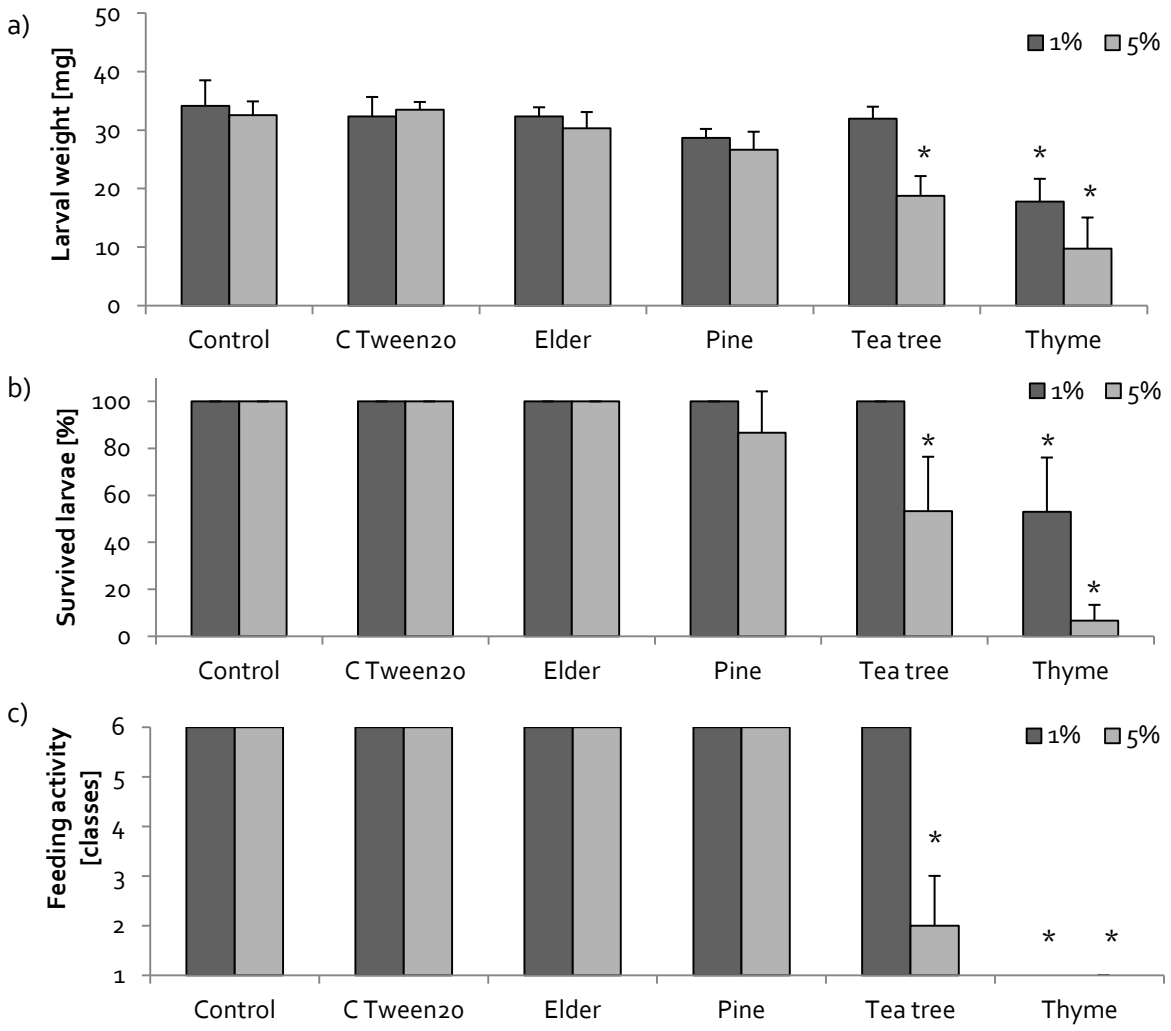


Figure 22 Feeding activity (a), survival (b) and weight (c) of *C. perspectalis* larvae exposed to leaf discs treated with selected oils. Asterisks indicate significant differences of means to the control (t-test).

Concentration response curve and lethal concentration (LC50)

A concentration response curve and effective lethal concentration (LC50) were further determined for *T. vulgaris* within 72 h bioassays. After 24 h, observed mortality rates already ranged between 7.5 and 72% for the different concentrations, after 48 h between 17.5 and 85% and after 72 h mortality rates between 18 and 92% could be determined (Figure 23). The concentration effect curve shows the influence of the *T. vulgaris* essential oil concentrations on larval mortality at 72 h and results refer to a distinct concentration response relation and parallelism of the calculated probit lines was confirmed (Figure 24). The LC50 value for *T. vulgaris* at 24 h was recorded as 2.7%, at 48h exposure time it was 1.3% and at 72 h it was 1.0%. The upper and lower 95% confidence interval at 72h ranged between 0.8% and 1.3% (Table 17).

Table 17 Larvicidal activity of *T. vulgaris* oil on *C. perspectalis* larvae after 24 h, 48 h and 72 h of exposure to treated leaf discs. Parameter given by probit analysis using linear maximum likelihood regression (df = 2).

time	chi²	intercept	slope (variance)	F (p)	LC_{50} [%]	95%-CI lower-upper
24 h	0.98	-0.96	2.20 (0.11)	86.93 (0.01)	2.7	2.2 - 3.7
48 h	1.26	-0.28	2.10 (0.10)	71.60 (0.01)	1.4	1.0 - 1.7
72 h	1.29	-0.06	2.44 (0.12)	77.28 (0.01)	1.1	0.8 - 1.3

Notes: Chi²-values: goodness of fit measure ($p > 0.05$); F-values: significance of slope different from zero; LC_{50}: effective Lethal Concentration; 95%-CI: lower and upper Confidence Interval, denoting significant differences if not overlapping

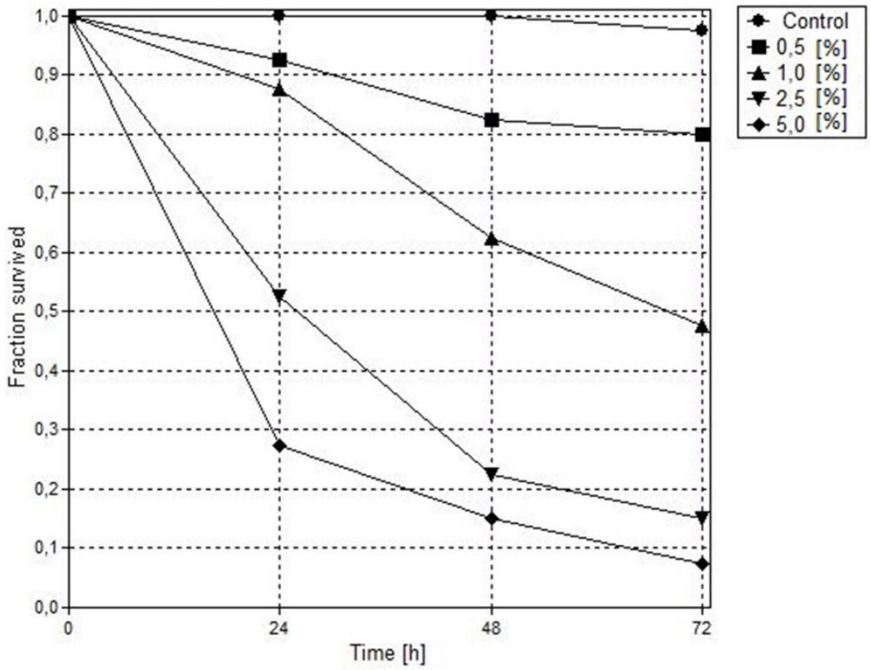


Figure 23 Mortality of *C. perspectalis* larvae at 24 h, 48 h and 72 h, exposed to different *T. vulgaris* oil concentrations (0%, 0.5%, 1%, 2.5% and 5%) on leaf discs.

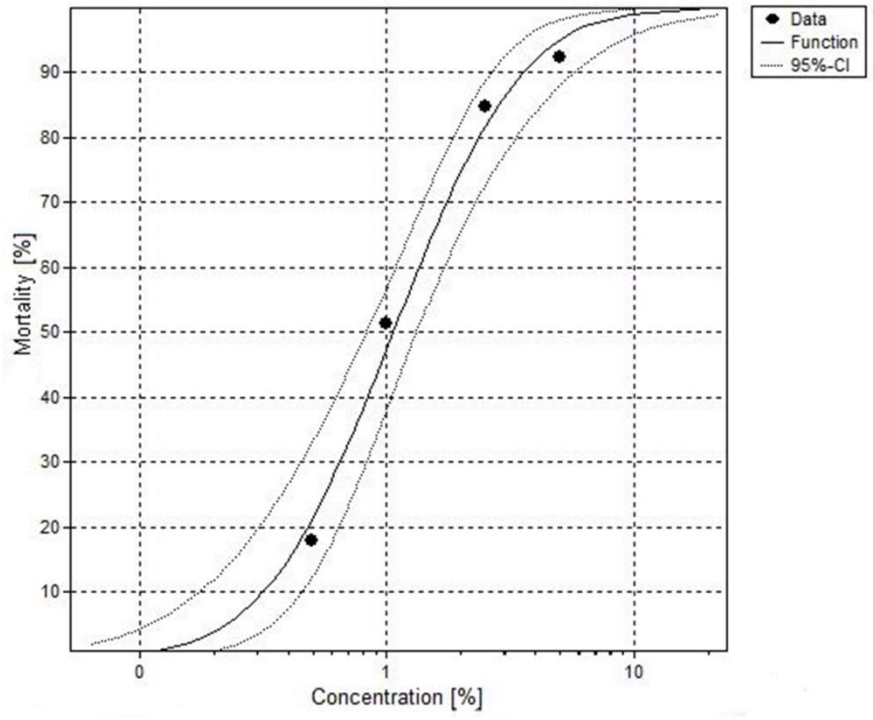


Figure 24 Concentration response curve, considering different concentrations of *T. vulgaris* oil to determine the influence of concentration on mortality of *C. perspectalis* 3rd instar larvae at a 95%-Confidence Interval 72 h after treatment.

Discussion

Up to date, applicable biological control strategies for regulating *C. perspectalis* in Europe are limited. Several methods were investigated but either they do not work well or have not been further tested, especially in field. Lee et al. (1997) tested the pathogenicity of the insect pathogen fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. GY1-17 against *C. perspectalis*, but it was not affected at a dose of 2.0 x 10^7 conidia/ml while mortality of *Plutella xylostella* L. (Lepidoptera: Plutelladae) larvae were 100% after 7 days at an equal treatment. Rose et al. (2013) found the baculovirus *Anagrapha falcifera* nucleopolyhedrovirus (AnfaNPV) to be infective for neonate *C. perspectalis* larvae at a median lethal concentration (LC_{50}) of 7.8 x 10^5 OBs/ml in a recent laboratory study but no plant protection product was subsequently developed. One nematode species (*Steinernema carpocapsae*) induced high mortality rates in laboratory studies (Choo et al. 1991b), but treatments of *C. perspectalis* larvae with nematodes were much less effective in the field (own investigation). There were several beneficials observed predating eggs or larvae in Asia and Europe (Wan et al. 2014), but investigations on the potential of parasitoid wasps belonging to the families Braconidae and Trichogrammatidae lead to the assumption that defense mechanisms occur in *C. perspectalis* eggs and larvae because parasitoids were not much successful (Zimmermann & Wührer 2010; Göttig & Herz 2016a). Using products based on *Bacillus thuringiensis* (strains *B. t. kurstaki* and *B. t. aizawai*) is currently the only effective biological control method but these preparations need to be sprayed at least twice against each generation and treatments require proper surveillance of the pest. In addition, there is a risk to the occurrence of resistance in the case of long-term applications. Furthermore, if not applied appropriately or timely, damage by larval feeding continues. Consequently, the demand for more environmentally and user friendly effective control methods is still urgent, especially on those preventing egg deposition and feeding.

Our results demonstrate an influence of essential oils and plant extracts on oviposition behavior of *C. perspectalis*. Seed oil of *S. nigra* was highly repellent to *C. perspectalis* females (RV = 0.94). Most known active substances of *S. nigra* are anthocyanins and other polyphenols obtained from the fruits and flower extracts (Dawidowicz et al. 2006; Stoilova et al. 2007) as well as lectins accumulated in the bark (Peumans & Van Damme 1998). They were mentioned to have medicinal properties (Sidor & Gramza-Michałowska 2015) as well as antioxidant qualities for body and skin, being mostly part of products of the food-, cosmetics- and pharmaceutical industries. Studies on the analysis of elder seed oil are rare. Investigations on the lipid and fatty acid composition were conducted (Dulf et al. 2013; Fazio et al. 2013) and a special lectin type was found to be present in the

seeds, which is a nonspecific glycoprotein containing 21% of carbohydrate and is rich in asparagine, serine, glutamine and glycine (Peumans et al. 1991). In comparison to essential oils and plant extracts there might be particular properties associated with physical features and also optical conspicuities. After application the foliage was covered with an obvious fatty spray layer. The leaves were luscious green, looked very healthy and were quite glossy and smooth and no phytotoxicity was observed. Therefore it is possible that females might be not only influenced by covering stimulants or the presence of an odor deterrent but by a change on the texture of the leaves' surface. Despite the special properties and its high oviposition repellency, there was no toxic impact on third instar larvae (including feeding activity, survival and weight) in bioassays conducted with treated leaf discs (Figure 22). This supported the assumption that the impact was not based on active substances in *S. nigra* seed oil, but rather the shift of surface texture. Also *T. vulgaris* demonstrated high repellency on females oviposition for the plant extract (RV = 0.78) and the essential oil (RV = 0.65) variant. Park et al. (2005) and Pavela et al. (2009) detected high efficiency of *T. vulgaris* oils against *Culex* spp. (Diptera: Culicidae). Oviposition was totally deterred from treated rainwater at very low concentrations of 0.02% and 0.01% and *T. vulgaris* oil effect repellency in a human forearm bioassay. In addition, *T. vulgaris* was reported to be very repellent against the Western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) at a concentration of 0.5% (Picard et al. 2012). In recent studies, thyme oil was tested against *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) flies. It caused high male mortality and reduced the number of responding flies to cotton wicks containing attractive raspberry juice (Renkema et al. 2016).

Larval performance of *C. perspectalis* was affected by *M. alternifolia* at 5% oil concentration and *T. vulgaris* at both tested concentrations (1% and 5%). *S. nigra* and *P. sylvestris* did not affect the feeding activity, survival or larval weight. *T. vulgaris* was more active than all other tested substances. Feeding was totally deterred, the weight was reduced and only a mean of 7% of larvae survived 72 h exposure of oil treated leaf discs. Because no feeding occurred, it appears that a kind of action might occur in the vapor phase. The LC_{50} was calculated to be 1% after 72 h. In the literature, *T. vulgaris* was repeatedly described to be very effective in terms of repellency and toxicity against various organisms in comparison to other tested oils. Yazdani et al. (2014) reported on high toxicity at 48 h (LC_{50} = 0.3%) and irreversible effects on key metabolic and feeding processes on 4^{th} instar larvae of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae), a related species to *C. perspectalis*. Also the cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) was shown to be affected by *T. vulgaris* oils via the leaf dip method. It was

one of the most toxic substances (LC_{50} = 0.5%) on 3rd instar larvae measured as 24 h mortality (Jiang et al. 2012). Moreover, thymol, the most active substance of *T. vulgaris*, affected many phytopathogenic bacteria and fungi (Kim et al. 2008; Kumar et al. 2008; Perina et al. 2015). At the same time their mammalian toxicity was proved to be very low (Kumar et al. 2008) and environmental persistence is short (Regnault-Roger et al. 2012). In comparison, our calculated LC_{50}-values (2.7% at 24 h, 1.4% at 48 h and 1% at 72 h) are up to five times higher. For an effective application in field, higher mortality rates are necessary. The LC_{90}-value is about 4% at 72 h. Thus, cost considerations become important, in the case of further investigations and the possibility of commercialization.

Despite the long history of global use for plant-based medicines and by the food and fragrance industry in Europe, botanical insecticides have not been widely embraced yet. There have been several problems on the commercialisation of botanical insecticides as plant protection products. Three main barriers were identified: (i) the scarcity of the natural resource; (ii) the need for chemical standardization and quality control; and (iii) difficulties in registration. For each of these there are also important cost considerations (Isman 2000, 2006). A number of plant essential oils (e.g. clove, spearmint and citronella) are approved as repellents but certainly there is still a lack of possibilities to use essential oils as a safe botanical preservative against different pests in the EU. The European Food Safety Authority is currently evaluating certain botanicals as low-risk active substances or basic substances as defined by (EC) Regulation No. 1107/2009 (Isman 2015; Marchand 2015). In the case of *T. vulgaris* oil, it was recognised in principle but no approval was passed yet for using it as an insecticide in Europe (EU 2010). In 2013, the active substance thymol was approved as fungicide (EU 2013). Since 2013, *T. vulgaris* has also been listed in Germany as a plant strengthening agent and some products can already be purchased on the market and used for plant strengthening (BVL 2017).

Our results demonstrate a first step on the possibility of using essential oils for preventing egg deposition on *Buxus* plants while influencing oviposition preference and protecting *Buxus* plants against feeding damage caused by *C. perspectalis* larvae. The next step would be to study field persistence and to optimise efficiency by suitable formulations and improved application rates.

General discussion

Challenges of regulating *Cydalima perspectalis* and saving *Buxus*

The establishment of *C. perspectalis* in Europe was facilitated by good preconditions found in its new environment. Due to the extensive plantings of *Buxus* in urban areas and favourable climatic conditions, that will probably be further enhanced by climate change, a large potential spreading area is available where the species is able to complete several generations per year (Dukes 2011; Nacambo et al. 2014; Göttig & Herz 2017). As a monophagous herbivore that is specialized in feeding on a plant genus containing alkaloids, *C. perspectalis* experiences only minor competition by other herbivores. Furthermore, alkaloids are accumulated in the larval body and may promote self-defense against natural enemies (Leuthardt et al. 2013). In addition, the limited awareness of the public and decision makers and the inadequacy of the legal framework certainly facilitated its establishment. Thus, eradication was already impossible at an early stage. All that remains to be done is the local control of already infested plants and the preservation of uninfected *Buxus* populations, in particular the naturally occurring stands.

In Germany, there are several chemical insecticides available and authorized for the current regulation of *C. perspectalis* on ornamental *Buxus* plants. But their use is often disliked or even prohibited, because ornamental *Buxus* plants are frequently part of private and public gardens as well as cemeteries and historical parks, where specific permissions must be complied with. Wan et al. (2014) reviewed control strategies in its native range of Japan and Asia and pointed out, that regulation in parks, green belts or nurseries relies mainly on chemical insecticides, such as pyrethroids. But due to the large-scale application of broad-spectrum insecticides, *C. perspectalis* has already developed some level of resistance in China. Furthermore, frequent applications of pyrethroids and neonicotinoids may cause severe negative effects on organisms that provide ecosystem services including pollination and natural pest control (EASAC 2015a). In the case of *Buxus*, bees and other pollinators are frequently visiting its flowers in spring (Kirk & Howes 2012). Besides the obviously lethal effect of chemical insecticides to non-target arthropods, sublethal effects on their physiology and behavior might be manifested like reductions in life span, development rates, fertility and fecundity as well as changes in sex ratio and behavior change in feeding, searching and oviposition (Stark & Banks 2003; Desneux et al. 2007).

The legal basis for plant protection in Germany and Europe focuses on an Integrated Pest Management (IPM) strategy, that should limit the application of chemical plant protection products to a necessary extent by using a combination of procedures which are under the primary

consideration of plant breeding and cultivation measures as well as biotechnical and biological methods (PflSchG 2012). Our investigations were focused on biological control methods with the intention of an easy and quick practicability and the aim of making chemical treatments avoidable, since their use is problematic in both planted and natural occurring *Buxus* stands. Biological control methods are of great advantage for regulating harmful lepidopteran pests and seem to be appropriate and promising for the eco-friendly regulation of *C. perspectalis*. Biological control comprises the use of biological plant protection products based on virus, bacteria, fungi, natural material, the use of pheromones, plant strengtheners and beneficials as well as grneral principles with the aim of reducing the population density of the pest organism (Franz & Krieg 1972; Schmutterer & Huber 2005). Advantages are the specific effects on targets and thus only slight impairment of non-target organisms; insignificant influence on ecosystems and no long-term effects on soil, water and air; little or no relevant residual traces occurring on plants as well as no waiting for bystander. In addition, biological control methods can close gaps in circumstances where chemical plant protection products may not be used. Furthermore, they might benefit the natural regulation of field populations by the preservation of natural enemies.

Various approaches on the biological regulation of *C. perspectalis* have already been examined in laboratory studies. Lee et al. (1997) tested the pathogenicity of the insect pathogen fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. GY1-17 but *C. perspectalis* larvae were not affected at a dose of 2.0 x 10^7 conidia/ml. Rose et al. (2013) found the baculovirus *Anagrapha falcifera* nucleopolyhedrovirus (AnfaNPV) to be infective for neonate *C. perspectalis* larvae at a median lethal concentration (LC_{50}) of 7.8 x 10^5 OBs/ml but no field trials were conducted and no plant protection product was subsequently developed. Despite Leuthardt & Baur (2013) mentioned an egg deposition preference for large-leaved *Buxus*, all varieties were affected during field observations, including the small-leaved variant *Buxus microphylla* Siebold & Zucc. that was the preferred host plant causing highest injury levels in its native range of Japan (Maruyama 1992). Plant breeding measures, such as the choice of resistant or tolerant varieties, are only suitable for the prevention of fungi infections by *Cylindrocladium buxicola* (LaMondia 2015; Shishkoff et al. 2015). Other well-known biological methods, such as mass sterilization and release of individuals seem to be out of the question, since no adequate mass breeding of the pest is possible so far. It would also be possible to net in *Buxus* plants for certain periods of time or cover the foliage by rock flour but such possibilities have not been sufficiently tested and lead to severe optical impairments of the plant.

The only effective alternative to chemical treatments is using products based on the bacteria *Bacillus thuringiensis* Berliner, the most widely used and intensively studied microbial insecticide.

These are specifically effective against lepidopteran larvae and its effectiveness on *C. perspectalis* is comparable to the application of chemical plant protection products, reaching up to 100% in the field. Furthermore, *B.t.* showed a higher persistence on *Buxus* foliage in field trials compared to other tested biological control agents (**Chapter IV**). Nonetheless, despite there are registered *B.t.* based products in Germany that are available on the market, problems linked to user restrictions and requirements for proper application are high. It is able to survive for several years after spraying in the soil of forest ecosystems and shows toxicity to non target species of several invertebrate taxa (Acarina, Nematoda, Collembola, Annelida, Hymenoptera) in the soil (Addison 1993). If treatment has to be done in public areas, they have to be closed for visitors until the spray deposit is completely dry (24 h) and provisions relating to water protection must be complied with, especially in nature reserves. Furthermore, as the only available biological control measure, the risk of resistance formation exists with frequent application (Schnepf et al. 1998). For example, the indian mealmoth *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) and the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) developed resistance to *B. t.* toxins in the field (McGaughey 1985; Tabashnik et al. 1990). Pesticide resistance is becoming an increasingly important factor in the case of using *B. t.* against lepidopteran species and the selection of biological control agents against pest species (Heckel et al. 2007; Kang et al. 2014).

Thus, the aim of this investigation was to set up an effective and eco-friendly method for the biological regulation of *C. perspectalis* that could be an alternative to chemical treatments and the frequent application of *B. t*. containing products. Our investigations were concretely focussed on the further development of a suitable monitoring system and research on the possible implementation of eco- and user friendly biological control methods, which are potentially eligible for all concerned *Buxus* stands. This represents a great challenge because of difficult growing conditions of both ornamental and natural *Buxus* stands. Ornamental *Buxus* populations are mostly based on a smaller amount of single plants but they are often in the vicinity of other *Buxus* plantings, especially in urban areas, each of different ownership structure and management responsibilities. Natural *Buxus* stands mostly appear in wide-range areas and are often located in slopy terrain that is difficult to access. The magnitude is often hard to comprehend and it is difficult to enter the whole area, such as in the case of the huge population in the Pyrenees. They are mostly part of nature reserves and the understory of forests where eco-friendly methods are urgent. Thus, our investigations were based on beneficials and biocontrol agents as well as on research on botanicals, considering potentially repellent and toxic effects to avoid egg deposition (see General introduction, Figure 5).

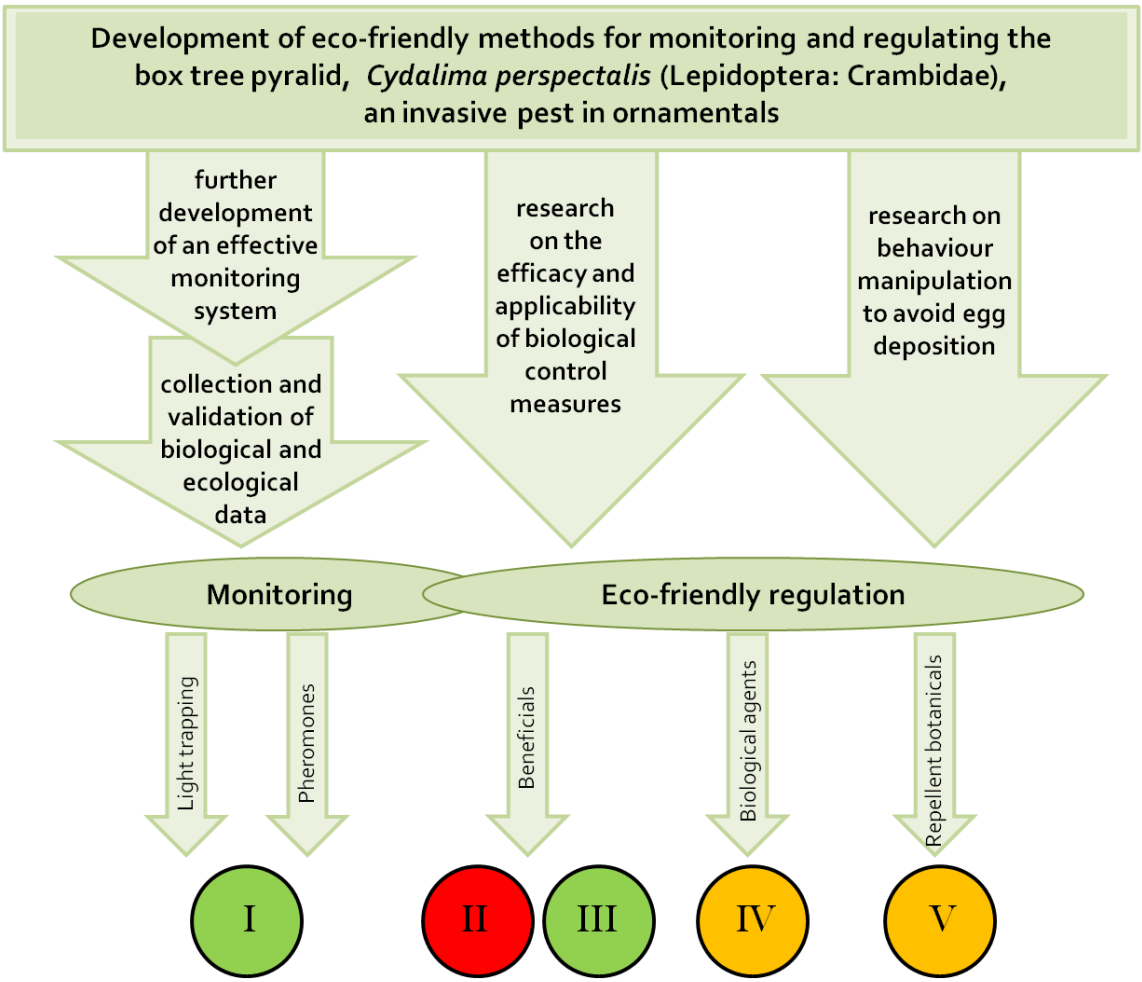


Figure 25 Schematic representation of thesis objectives, assignment of manuscript chapter (see Figure 5) and the usability level (red, orange, green: low to high) of components for the biological control of *C. perspectalis*.

Monitoring is an important component when dealing with invasive species, according to assess the spread, population levels and the seasonal flight activity of the target per year. Reliable monitoring is based on a species specific, effective and easy applicable trapping system including the correct pheromone compounds (Valles et al. 1991). Therefore own investigations had been previously conducted on the improvement of the *C. perspectalis* female pheromone composition. Based on the results of Kawazu et al. (2007) and further analyses, an improved and well trapping pheromone was developed in cooperation with the company Pherobank B.V. (Wijk bij Duurstede, Niederlande). Its efficiacy was experimental proofed by field evaluations that were conducted simultaneously in several European countries (not yet published and not mentioned in this thesis). Using this pheromone composition, our study on observations of the seasonal flight activity of *C. perspectalis*

populations in Hessia (Göttig & Herz (2017), **Chapter I**) demonstrated a well improved and adapted trap system for monitoring *C. perspectalis*. It was able to attract males in a large amount and to identify the seasonal flight activity that lead to conclude two completed generations per year. Furthermore, it supports an early detection of egg deposition and larval feeding phases of occurring populations on a local scale. Additionally, the documentation of the regular occurrence of melanic moths provided useful insights into the biology of this invasive organism. Pheromone trapping play an important role for monitoring within adapted biological management programs. Systems were developed and well established for common crop pests as codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) in orchards of apple *Malus domestica* Bordhausen, where also successful mating disruption strategies took place (Knight et al. 2013) and the European corn borer *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae) on sweet corn (Laurent & Frérot 2007). In the case of *C. perspectalis,* an effective monitoring is now possible and can be highly useful in both ornamental and natural *Buxus* stands.

Beneficial organisms for the purpose of crop protection are usually invertebrate animals mostly belong to the phylum Arthropoda or Nematoda and they are acting as predators, parasitoids or parasites. Common natural enemies of *C. perspectalis* were observed in Asia and Europe, such as Diptera (Tachinidae), Hymenoptera (Braconidae, Chalcididae, Encyrtidae, Ichneumonidae), Heteroptera (including the family Anthocoridae) and Coleoptera (Coccinellidae: *Harmonia* spp.) as well as predators such as Thysanoptera (Aeolothripidae) and spiders (Araneae). These predators occur naturally in *Buxus* habitats and were observed to prey on *C. perspectalis* eggs, larvae or pupae but their impact on populations remain low and mostly unknown (Wan et al. 2014). The native larval parasitoid *Bracon brevicornis* Fabricius (Braconidae) was tested on its efficacy against *C. perspectalis* in Germany but the results lead to the assumption that *C. perspectalis* larvae defended parasitization, since the parasitoid species was not able to complete its development in it (Zimmermann & Wührer 2010). Our investigation on prey acceptance and location of *C. perspectalis* eggs (Göttig & Herz (2016a), **Chapter II**) demonstrated, that egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) may also have to deal with egg defence mechanisms that may impede higher parasitization and have not been fully understood. Trauer-Kizilelma & Hilker (2015) demonstrated improved defence efficiency in *Manduca sexta* L. (Lepidoptera: Sphingidae) eggs by an enhanced immune response to parasitisation by *T. evanescens*. During our acceptance tests in the laboratory, *T. pintoi* had the highest number of egg encounters and female activity on *C. perspectalis* eggs but *T. dendrolimi* females were most able to locate the host eggs and to oviposit and produce successfully on them. Further study is needed to

clarify the suspected role of the potentially egg and larval defensive mechanisms that may impede higher parasitism and prey on *C. perspectalis*. *Trichogramma* wasps are already been used successfully for years within biological plant protection strategies. There are several effective systems developed for their release in field as well as in the greenhouse. The release of *T. brassicae* against the European corn borer *O. nubilalis* in field has the largest share of 20000 ha in Germany (Jehle et al. 2014). Other species are adapted and very successful against the codling moth *C. pomonella* L. and the plum fruit moth *Grapholita funebrana* Treitschke (Lepidoptera: Tortricidae) in private gardens (Hassan 1989; Zimmermann 2004, 2007). In the case of *C. perspectalis* no adapted *Trichogramma* species was found yet that can parasitize *C. perspectalis* in an adequate amount and might be sufficient for a potential inundative release in field. Further research on the potential of other *Trichogramma* strains and the behavior of *Trichogramma* females is actually carried out in France (Tabone et al. 2015). Currently, there is no possibility given for an efficient regulation by *Trichogramma* wasps, neither in ornamental nor in natural *Buxus* stands.

In general, there are four different possibilities to use beneficials for the regulation of invasive pests. These include the conservative, inoculative, inundative and classic strategy. Within the conservative strategy, already occurring natural enemies are preserved to conserve or optimize the functional biodiversity in the concerned habitat. Inoculative and inundative strategies are dealing with the establishment or periodic release of laboratory reared native species. For the classic strategy, specialized non-native antagonists are introduced from the native region of origin to establish and decrease pest populations in long-term scales (IOBC 2012). In China, one specific and well adapted egg-larval parasitoid *Chelonus tabonus* Sonan (Braconidae) was found and might be the only possible candidate for classical biological control, but its impact on *C. perspectalis* populations is unknown and its host specificity and potential introduction into Europe has to be further investigated (Wan et al. 2014). Our study on the prey acceptance and location of *C. perspectalis* eggs by the commercially available beneficials *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and *Orius majusculus* Reuter (Heteroptera: Anthocoridae) (**Chapter III**) clarified, that both tested species preyed on *C. perspectalis* eggs and might be considered as useful supporting tools in the biological control of *C. perspectalis*. *C. carnea* larvae are the more voracious predators, but their natural occurrence on *Buxus* plants is rather low. Therefore, consideration may be given for an augmentation by an inundative release of larvae of these native and commercially available beneficials, especially in the local scales of ornamentals. Because of the natural occurrence of many predatory beneficials on *Buxus* plants, their protection and conservation might be more profitable

than their release. The study underlined the importance to avoid chemical treatment in both ornamental and natural *Buxus* stands.

Our investigation on the susceptibility of *C. perspectalis* to the biological control agents Neem (NeemAzal®-T/S) and Entomopathogenic nematodes (Nemastar®) **(Chapter IV)** pointed out, that *C. perspectalis* larvae are susceptible to both agents in the laboratory but agents were much less reliable in their efficacy compared to the constant effectiveness of *B. t*. applications in field. Neither the application of NeemAzal®-T/S nor the use of Nemastar® caused mortality rates comparable to the effectiveness of plant protection products based on the bacteria *B.t.*in field trials. This also applied to the persistence of products on the foliage. Reasons for the difficulties regarding the application of nematodes are less to be found in the general susceptibility than in the application technology. Despite the larvae are highly affected by *S. carpocapsae* in laboratory bioassays (Göttig 2012), the rate of mortality in field was fluctuating. Nematode success to penetrate host species is strongly related to weather conditions, the application technique and easy accessibility of the host on the plant foliage, in contrast to common soil applications. Factors like the UV irradiation and temperature, as well as the relative humidity play a decisive role in foliar application and the persistence of EPN in field (Glazer 1992; Smits 1996; Arthurs et al. 2004). As beneficial agent, the application of EPN requires no permission and there are no user restrictions in many countries.

In the case of neem, the time until larvae respond to the treatment was highly extended. None of the tested concentrations (1-5 ml/ l) led to mortality earlier than 6 days after first ingestion of treated leaf discs. There was only little variation in the response on the different concentrations and ingestion of treated leaf discs caused feeding stop and larval mortality within two weeks in laboratory bioassays. Field mortality was difficult to measure, because of the extended responding time. Larvae were vital and mostly still feeding 7 days after the application when the trials were finished in field. Thus, the use of Neem products against *C. perspectalis* proofed to be inappropriate for an effective control. The use of these products as biological control agents to regulate *C. perspectalis* would only be possible if individual feeding damage and proper surveillance will be considered in order to be capable of repeating the application timely. More frequent and timely applied treatments may improve efficacy and a combination with further measures ought to take place. That is also the reason, why these strategies are not suitable to protect or save the extended natural *Buxus* stands and being more appropriate for the application in local scales like private gardens or in the urban green areas.

New approaches dealing with moth behavior manipulation to protect *Buxus* plants from egg deposition are slightly promising. Female moths are using visual, contact and olfactory cues of the host plant to find an adequate oviposition site, which all can be manipulated. In our study on repellent and toxic properties of plant oils and extracts on *C. perspectalis* **(Chapter V)**, the application of *Thymus vulgaris* L. essential oil reduced female egg deposition and larvae were significantly susceptible to treated and exposed leaf discs including feeding deterrence, survival and larval weight. In addition, recently Molnar et al. (2017) detected three compounds in the larval frass of *C. perspectalis*, that have a significant oviposition-repelling effect when a blend was used containing a synthetic mixture of these volatile compounds in the laboratory. In general, the repellent effect of a substance causes the target pest to avert from the source of the odor and thus, from the treated plant (Dethier et al. 1960). In addition to the prevention of egg deposition, this can facilitate an orientation towards an attractive fragrance. Therefore, further possible applications are conceivable in which repellents can serve as a helpful push component in the sense of a push-pull system (Cook et al. 2007). Nevertheless, there are some problematic factors in the use of essential oils within plant protection strategies. There are comparatively high costs (Young 2004), the high volatility of the substances (Turek & Stintzing 2013), the potential phytotoxicity (Amri et al. 2013) as well as possible side effects on non-target organisms. Nonetheless, *T. vulgaris* has been listed in Germany as a plant strengthening agent and some products can already be purchased on the market and used for plant strengthening in ornamentals. In addition, these approaches seem to be innovative and promising supporting tools for the prevention of both ornamental and natural *Buxus* stands. But further investigations on the efficacy of essential oils in field as well as field applicable methods and formulations are required.

The development of a strategy for the local control of already infested *Buxus* plants and the preservation of uninfected populations, in particular the naturally occurring stands, turned out to be a difficult challenge. The investigated components were assessed to be differently usable for the biological control of *C. perspectalis* (Figure 25) and appropriate modes of action were integrated in *C. perspectalis* seasonal life cycle for precise scheduling. Findings of **Chapter I** were highly useful to detect the seasonal moth flight activity and be able to provide the common seasonal phenology of *C. perspectalis* as well as general indications for the proper timing of control methods (Figure 26).
In addition, the improved pheromone system was found to be suitable for an exact individual monitoring of particular *Buxus* stands on a local scale, which is required for a proper surveillance and early detection of *C. perspectalis* populations.

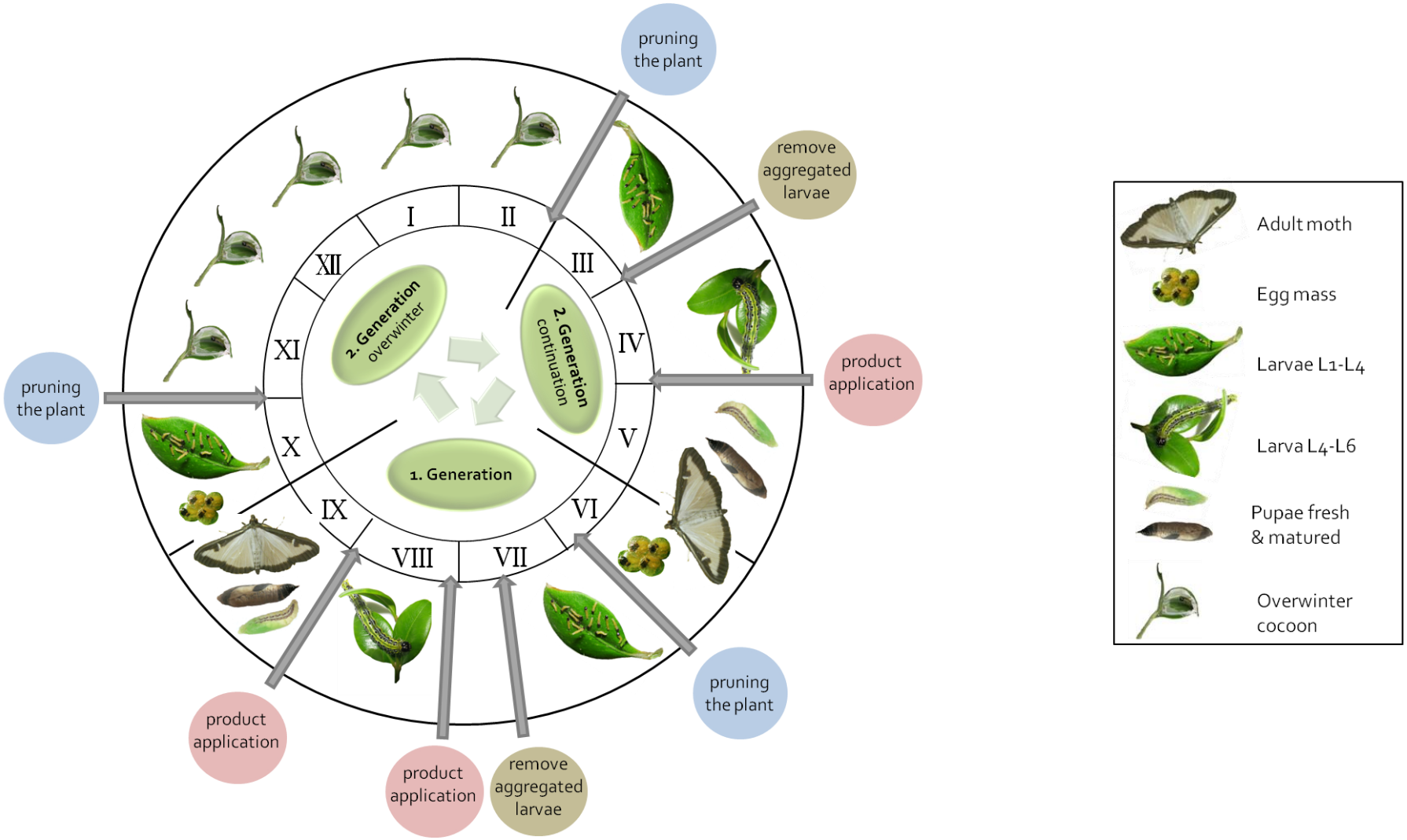


Figure 26 Seasonal phenology of *C. perspectalis* (see Figure 4) and general indications for the eco-friendly regulation.

In the case of the presence of *C. perspectalis* first steps could be sufficient to prevent larval hatch, avoid aesthetic damage and defoliation of *Buxus* by larval feeding. Common physical measures are considered to be necessary and helpful as well as already mentioned by plant protection services: (1) pruning the plant to remove winter cocoons and egg masses (2) removal of larvae by hand that is mostly effective if timely conducted while small larvae are feeding aggregated and (3) washing down the plants with a strong water jet and remove larvae that are coming off. The use of *Trichogramma* wasps is currently no suitable control tool since no specialized *Trichogramma* species had been found yet for a practicable inundative release to parasitize *C. perspectalis* eggs in the field **(Chapter II)**. Findings of **Chapter III** suggest that the use of measures should be limited to a control by biological means since the conservation of natural enemies as *O. majusculus* and the augmentation by an inundative release of *C. carnea* can be considered as supporting tools for the biological control of *C. perspectalis*. Field population densities can potentially be influenced by egg predators, which should be released during the egg deposition phases. However, neem-based products and, above all, nematodes can support larval regulation under certain conditions and in combination with other means. Their use is only possible in smaller areas, if individual feeding damage and proper surveillance will be considered (**Chapter IV**). New approaches dealing with moth behaviour manipulation by reppelents like *Thymus vulgaris* essential oil are promising tools since the concept leads to the preservation of unaffected *Buxus* plants **(Chapter V)**. But up to date, none of the tested control options alone provide the opportunity for an effective, eco- and user friendly regulation of *C. perspectalis*. Applied biological control strategies for regulation *C. perspectalis* in Europe are still limited. Several methods were investigated, but they did not work or have to be further investigated. The application of products based on *B. thuringiensis* is still the only highly effective and available biological control method.

The present situation requires an extensive knowledge of the biology and ecology of *C. perspectalis* or even a professional advice to be able to control the pest ever reasonably. This can already be a challenge in the ornamental plant sector and can hardly be realized in the case of extensive natural Buxus stands. The difficult conditions as well as the additional threat by the occurrence of the harmful fungus *Cylindrocladium buxicola* (Göttig & Herz 2016b), see General introduction, Box 2) are additionnally problematic factors for the protection of *Buxus.* The urgent need of long-term investment of time and money to protect *Buxus* and control *C. perspectalis* often leads the affected parties to remove injured *Buxus* plants and replace with alternative plantings that require only low maintenance. In conclusion, an eco-friendly regulation of the invasive Box tree moth *C. perspectalis* still remains a major challenge for all concerned parties, even 10 years after the introduction.

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List of special abbreviations

aa	adhesive agent
BOU	*Trichogramma bourarachae*
BRA	*Trichogramma brassicae*
BT	*Bacillus thuringiensis*
BTa	*Bacillus thuringiensis aizawai*
BTk	*Bacillus thuringiensis kurstaki*
C	Control
CAC	*Trichogramma cacoeciae*
COR	*Trichogramma cordubensis*
cw	calendar week
DA	Darmstadt
DAT	Days after treatment
DEN	*Trichogramma dendrolimi*
DFI	Days of further incubation
EPN	Entomopathogenic Nematodes
EVA	*Trichogramma evanescens*
IJs	Infective Juvenils (EPN)
LC	Lethal concentration
LT	Light trap
NA	NeemAzal-T/S
NER	*Trichogramma nerudai*
PIN	*Trichogramma pintoi*
PT	Pheromone trap
RV	Repellency value
SE	Seligenstadt
wa	wetting agent

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Curriculum vitae

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Publications

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GÖTTIG S. & HERZ A. 2014**:** The box tree pyralid *Cydalima perspectalis*: New results of the use of biological control agents and pheromone traps in the field. Deutsche Phytomedizinische Gesellschaft (DPG). Journal of Plant Diseases and Protection 121(2): 98-99. 32. Tagung des Arbeitskreises „Nutzarthropoden und Entomopathogene Nematoden", Darmstadt

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HERZ A. & GÖTTIG S. 2015: Eco-friendly regulation of the Box tree pyralid *Cydalima perspectalis.* IUFRO conference: Population dynamics and integrated control of forest defoliating and other insects, Sopot (Poland)

HERZ A., FEIERTAG S., GÖTTIG S. & OELLERS F.M. 2011: Der Buchsbaumzünsler, *Cydalima perspectalis* (Walker 1859) (Lepidoptera, Pyralidae) in Südhessen - Vorkommen, Ausbreitung und Aussichten für eine biologische Regulierung mit Nützlingen. Entomologentagung / Deutsche Gesellschaft für allgemeine und angewandte Entomologie, Berlin

Poster

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Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den 14.07.2017